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Protection of Atlantic salmon (*Salmo salar* L.) against an experimental infection of *Aeromonas salmonicida* ssp. *achromogenes*

BJARNHEIDUR K. GUDMUNDSDÓTTIR* AND BERGLJÓT MAGNADÓTTIR

Institute for Experimental Pathology, University of Iceland, Keldur, IS-112 Reykjavík, Iceland

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To assess the protective role of cell-associated and extracellular antigens of *Aeromonas salmonicida* ssp. *achromogenes*, Atlantic salmon were immunised either with detoxified extracellular products (d-ECP), formalin killed cells (f-Cells) or a mixture of both. In an experimental challenge ECPs were found to elicit better protection than whole bacteria. The protection strongly correlated with the detection of antibodies directed against a toxic metallo-caseinase, AsaP1, in fish sera. Passive immunisation of Atlantic salmon with rainbow trout or rabbit anti-AsaP1 antisera conferred significant protection against challenge with a virulent *A. salmonicida* ssp. *achromogenes* strain. In an equivalent experiment neither rainbow trout nor rabbit antisera containing antibodies to the A-layer protein of *A. salmonicida* were found to be protective for passively immunised Atlantic salmon. The results demonstrate the importance of humoral antibodies and neutralisation of bacterial toxins in protecting Atlantic salmon against *A. salmonicida* ssp. *achromogenes*

Key words: *Aeromonas salmonicida* ssp. *achromogenes*, extracellular products, metallo-caseinase, AsaP1, A-layer protein, *Salmo salar,* vaccination, passive immunisation, protection.

I. Introduction

A number of cell-associated and extracellular virulence factors of the Gram-negative bacterium *Aeromonas salmonicida* have been described (reviewed in Austin & Austin, 1993). Atypical (including ssp. *achromogenes*, ssp. *masoucida* and ssp. *smithia*) and typical (ssp. *salmonicida*) strains of *A. salmonicida* have been reported to share cell-associated antigens such as the A-layer protein, the LPS component, iron-regulated outer membrane proteins and porins (Evenberg *et al.*, 1985; Pyle & Cipriano, 1986; Chu *et al.*, 1991; Hirst & Ellis, 1994; Lutwyche *et al.*, 1995), but exotoxins produced by typical and atypical strains, including ssp. *achromogenes*, are found to be of a different nature (Gudmundsdóttir *et al.*, 1990; Lee & Ellis, 1990; Ellis, 1991; Gudmundsdóttir, 1996).

*Author to whom all correspondence should be addressed at: Institute for Experimental Pathology, University of Iceland, Keldur, v/Vesturlandsveg, IS-112 Reykjavík, Iceland.

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A knowledge of the host immune response is of prime importance in understanding the pathogenic mechanism of a bacterium, and for inducing prophylaxis. The majority of the literature on immunity to *A. salmonicida* antigens reports studies in response to ssp. *salmonicida* antigens in salmonids. Furthermore, commercially available *A. salmonicida* vaccines are produced from typical *A. salmonicida* strains for prevention of furunculosis in salmonids. However, as diseases caused by atypical strains are of emerging importance worldwide the prospects of their control by vaccination need to be considered.

In Icelandic aquaculture, infections due to *A. salmonicida* ssp. *achromogenes* are causing serious problems in cultivated salmonids on fish farms using brackish water for rearing and in captive cod of wild origin cultivated in sea cages. Furthermore, the bacterium has been isolated from wild fish of many species and the susceptibility of halibut to the bacterium has been shown by experimental infection (Gudmundsdóttir *et al.*, 1996).

Vaccine-induced protective immunity against atypical *A. salmonicida* in carp (*Cyprinus carpio* L.) has been reported by Evenberg *et al.* (1988) and the authors have obtained protection in Atlantic salmon vaccinated with experimental *A. salmonicida* ssp. *achromogenes* bacterins (Gudmundsdóttir *et al.*, 1995). Studies of passive immunisation of fish with immune sera from fish or rabbits have shown that humoral factors can protect fish against *A. salmonicida* ssp, *salmonicida* infection (Spencer & Fryer, 1965; Cipriano, 1982; McCarthy *et al.*, 1983; Olivier *et al.*, 1985; Ellis *et al.*, 1988; Marquis & Lallier, 1989; Hirst & Ellis, 1994). The aim of the present study was to compare protection in salmon actively immunised either with *A. salmonicida* ssp. *achromogenes* whole cells, extracellular products or a mixture of both antigens. Furthermore, to investigate, by passive immunisation, if antibodies to two important virulence factors are involved in the protection of Atlantic salmon (*Salmo salar* L.) against experimental infection with *A. salmonicida* ssp. *achromogenes*. The respective antisera were against the cell-associated A-layer protein and AsaP1, an extracellular lethal toxic caseinase of *A. salmonicida* ssp. *achromogenes*.

II. Material and Methods

BACTERIA AND BACTERIAL ANTIGENS

Two different strains of *A. salmonicida* were used in this study: 265-87, originally isolated from diseased Atlantic salmon and 265-87-1, an A-layer negative mutant of strain 265-87. The strains are non-motile, facultative anaerobic Gram-negative short rods that are homogeneous with the type strain for *A. salmonicida* ssp. *achromogenes*, NCMB 1110 (National Collection of Marine Bacteria), as regards all the characteristics listed in Table 1, except for the Voges-Proskauer reaction, and are thus classified as *A. salmonicida* ssp. *achromogenes*. The biochemical reactions were performed using conventional test media. Incubations were for 7 days at 20°C and negative reactions were repeated once before confirmation. The strains secrete proteases comparable with those of the type strain, including a toxic caseinolytic metallo-protease, AsaP1 (Gudmundsdóttir, 1996). Strain 265-87 possesses the

Table 1. Comparison of several characteristics of the type strain of *A. salmonicida* ssp. *achromogenes* (NCMB 1110) and the strain used in the study (265-87). Plus (+) indicates a positive reaction; minus (−) a negative one

Characteristic	265-87	NCMB 1110
Autoagglutination	+	+
Motility	−	−
Catalase	+	+
Oxidase	+	+
Glucose fermentation	+	+
Arginine dihydrolase	+	+
Lysine decarboxylase	−	−
Ornithine decarboxylase	−	−
Indole production	+	+
Voges-Proskauer	−	+
Esculine hydrolysis	−	−
Gas from glucose	−	−
H_2S production	−	−
Acid from		
Sucrose	+	+
Mannitol	+	+
Salicin	−	−
Resistance to		
Ampicillin 33 μg	R	R
Cephalothin 66 μg	R	R
Production of		
Brown pigment	+*	(+)*
Haemolysin	−	−
Gelatinase	+	+
Caseinase (AsaP1)	+	+

*Pigment after 3 days at 22° C, R=resistant.

two well known cell-associated virulence factors, the lipopolysaccharide (LPS) and the A-layer. Strain 265-87-1 lacks the A-layer but secretes the A-layer protein (Magnadóttir *et al*., 1995). Standard bacteriological techniques were used to examine morphological, physiological and biochemical criteria of the strains (MacFadden, 1983; Frerichs, 1984). Incubations were performed at 22°C for 7 days. Antibiotic sensitivity tests were performed using Neo-Sensitabs (Rosco). An examination of extracellular proteases was performed as described by Gudmundsdóttir (1996). Stock cultures were stored in brain heart infusion broth (BHIB, Oxoid) containing 15% glycerol at −80°C.

Bacterial antigens were prepared from strains 265-87 and 265-87-1. Cultures were made with the cellophane overlay method of Liu (1957) using BHI agar and incubation at 22°C for 72 h. The culture was washed from the cellophane with a minimal volume of phosphate-buffered saline (PBS, pH 7·2) and centrifuged. The supernatant (extracellular products; ECP) was desalted on a Sephadex G-25M (Pharmacia PD-10) column and filtered (Millepore, 0·22 μm). In order to inactivate toxic enzyme activity, the ECP was treated by dialysing overnight at 4°C in 25 volumes of 10 mM OPA (1,10-phenanthroline), 1 mM

phenyl methyl sulphonyl fluoride in PBS, followed by addition of formalin to a final concentration of 2% (w/v) and incubation for 7 days at 22°C. Following inactivation, the detoxified-ECP (d-ECP) was dialysed against 200 volumes of distilled water for 24 h at 4°C and concentrated by dialysis in polyethylene glycol at 4°C to approximately one fourth of its original volume. Protease and cytotoxic activities of the d-ECP were determined by caseinolysis and cytotoxicity assay using cultured rainbow trout gonad cells, as described by Gudmundsdóttir *et al.* (1990). Neither proteolytic nor cytotoxic activities were detected in d-ECP.

Cells were killed with 2% (w/v) formaldehyde and incubation at 22°C for 24 h. Formaldehyde-treated cell suspensions were washed three times by centrifugation (2000 $g \times 30$ min) in PBS (f-Cells).

The 20-kDa metallo-protease, AsaP1, was isolated from an ECP preparation of isolate 265-87 as previously described (Gudmundsdóttir *et al.*, 1990). Briefly, anion-exchange chromatography was followed by gel-filtration using Mono Q HR 5/5 and Superose 12 HR 10/30 columns, respectively, and the FPLC system from Pharmacia. Proteolytic activity in peak fractions was assayed using azocasein as a substrate. Detoxification of AsaP1 (d-AsaP1) was performed by dialysing overnight at 4°C in 25 volumes of 10 mM OPA and heating for 15 min at 55°C. Caseinase activity was completely inhibited by this treatment.

In order to detect toxic properties of the ECP and AsaP1 antigens, fish were injected intraperitoneally (i.p.) with 0·1 ml of detoxified preparations.

The 50-kDa A-layer protein was isolated from the ECP of strain 265-87-1 using anion-exchange chromatography and gel-filtration as previously described by Magnadóttir *et al.* (1995).

Protein concentrations of antigen preparations were determined by the Bradford protein assay (Bradford, 1976) using bovine serum albumin (BSA) as standard and stored in aliquots at -80°C until used.

FISH HANDLING

The fish were kept in 400 l tanks with continuously flowing well water at an ambient temperature of 10 ± 3°C and fed commercial dry pellets with an automatic feeder. Prior to initiation of experimental work the fish were acclimatised for 1 week. Before any treatment the fish were marked with Alcian blue dye. During injection and bleeding procedures the fish were anaesthetised by immersion in benzocaine at a concentration of 40 mg l^{-1}.

ACTIVE IMMUNISATION AND CHALLENGE OF ATLANTIC SALMON

Five test-groups, each containing 60 Atlantic salmon fingerlings with an average weight of 30 g, were used for active immunisation. The fish were equally distributed into two tanks. The vaccines, as well as the control preparations were administered by i.p. injections of 0·1 ml of each antigen emulsified with an equal volume of Freund's complete adjuvant (FCA; Sigma). At day 60, the fish received a booster injection of the same antigen in Freund's incomplete adjuvant (FIA; Sigma). *A. salmonicida* ssp. *achromogenes* antigens used for active immunisation of Atlantic salmon are listed in Table 2.

Table 2. The effect of active immunisation of Atlantic salmon measured 90 days post-vaccination. Humoral antibodies to ECP of strain 265-87-1, which secretes the A-layer protein, were measured by ELISA in a pool of sera (diluted 1/200) from control (PBS, adjuvant) and vaccinated Atlantic salmon (d-ECP, f-Cells, d-ECP/f-Cells). Relative percent survival (RPS) was calculated after an i.m. infection challenge by *A. salmonicida* ssp. *achromogenes*, strain 265-87

Antigen used in vaccination	Dose/fish μg protein	ELISA value* *N*/group=5	% mortality	RPS *N*/group=60	χ^2 (df=1)
PBS	—	0·21	86	—	
Adjuvant	—	1·20	57	34	0·0011
d-ECP	80	1·80	0	100	<0·0001
f-Cells	200	1·55	35	59	<0·0001
d-ECP/f-Cells	40/100	3·50	17	80	<0·0001

*ELISA value at $O.D._{492}$.

Caudal blood samples from five fish in each test-group were taken on day 90. Blood from each treatment group was pooled, allowed to clot at 15°C for 4 h and 4°C overnight, centrifuged at 2000 *g* for 10 min and serum collected.

Challenge was performed 90 days after the primary immunisation by intramuscular (i.m.) injection, lateral to the dorsal fin, of 10^4 colony forming units (cfu) of isolate 265-87. Density of bacterial solutions was estimated by the drop (25 μl) plate method of Miles & Misra (1938) using incubation on blood agar. Deaths were recorded daily for 4 weeks. Mortality caused by *A. salmonicida* ssp. *achromogenes* was confirmed bacteriologically by re-isolation of the bacterium from kidney samples.

Relative percent survival (RPS) was calculated according to Amend (1981). Mortality in different groups was compared by chi-square analysis.

DOUBLE SANDWICH ANTI-ECP ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The enzyme-linked immunosorbent assay (ELISA) has been described in detail (Magnadóttir & Gudmundsdóttir, 1992). Briefly, ELISA trays (Nunc) were coated overnight with 100 μl per well of strain 265-87-1 ECP, which secretes the A-layer protein (10 μg protein ml^{-1}), blocked with BSA and overlaid with 100 μl of test sera in two-fold serial dilutions, from 1/100–1/409 600, in PBS containing 0·05% Tween-20. After an overnight incubation at 4°C, bound antibody was detected. The rabbit antibody was detected with peroxidase-labelled swine antibody to rabbit immunoglobulins (Dako) and the fish antibody with a polyclonal mouse antibody to salmonid IgM (prepared in our laboratory) followed by peroxidase-labelled rabbit antibody to mouse immunoglobulins (Dako). Incubations were performed for 1 h at 37°C. Colour was developed with 1,2-phenylenediamine dihydrochloride (OPD) substrate (Sigma) for 30 min at 22°C. Between each step, wells were washed extensively with PBS containing 0·05% Tween-20. Optical density was read at 492 nm. The titre was determined as the reciprocal value of the highest dilution that gave an optical density reading of about 0·2 at 492 nm against a negative control blank with the serum replaced by PBS containing 0·1% Tween-20 (PBS-T).

Table 3. Control and antisera used to determine efficacy of passive immunisation to protect Atlantic salmon against experimental challenge with *A. salmonicida* ssp. *achromogenes*, strain 265-87. The ECP of an A-layer negative mutant of strain 265-87 (265-87-1) which secretes the A-protein was used as an antigen in the ELISA test

Antigen (μg protein ml^{-1}) dose	Animal injected	Serum designation	ELISA
	(not immunised)	NRS	800
AsaP1 (100)	Rabbit	Ranti-AsaP1	51 200
A-protein (1000)	Rabbit	Ranti-A-protein	>409 600
	(not immunised)	NTS	200
AsaP1 (100)	Rainbow trout	Tanti-AsaP1	800
A-protein (1000)	Rainbow trout	Tanti-A-protein	6400

ANTISERA AGAINST AsaP1 AND A-LAYER PROTEIN PREPARED IN RAINBOW TROUT AND RABBIT

Antigen preparations used for immunisations are listed in Table 3.

Trout antiserum was raised in rainbow trout (*Oncorhynchus mykiss*), weighing approximately 300 g. Immunisations, as well as control injections, were administered by i.p. injection of 0·2 ml of each antigen emulsified in an equal volume of FCA. Three fish were in each treatment group. After 60 days the fish received a booster injection of the same antigen in FIA. Antisera were isolated as described above and serum samples from each treatment group pooled. Normal trout serum (NTS) was collected from the trout before immunisation.

Rabbit antiserum was raised in an adult New Zealand white rabbit by several subcutaneous injections of the antigen emulsified with an equal volume of FCA. On days 20 and 34 the rabbit received a booster of the same antigen in FIA. The rabbit was bled 20 days later and serum extracted as described above and stored at -20°C until used. Normal rabbit serum (NRS) was collected from the rabbit before immunisation.

PASSIVE IMMUNISATION AND CHALLENGE OF ATLANTIC SALMON

Trout and rabbit antisera were heated at 54°C for 15 min in order to inactivate the complement factors. Atlantic salmon fingerlings with an average weight of 40 g were passively immunised by i.m. injection of 0·1 ml of heated antiserum and an i.p. injection of 0·1 ml of untreated antiserum. Control fish received either normal serum or PBS instead of immune serum.

Passaged strain 265-87 was cultivated in BHIB at 22°C. After incubation for 16 h, the cells were washed once and diluted with sterile PBS. The cultures were checked for purity and cfu confirmed by plate counting.

Following passive immunisation and control injections the fish were challenged by i.m. injection of 0·1 ml bacterial suspension (strain 265-87). Ten-fold dilutions of the bacteria in PBS (containing 10^2–10^9 cfu ml^{-1}) were used for challenge and a group of six fish from each of the seven treatment groups injected with each dilution. Deaths were recorded daily for 4 weeks.

To estimate the effect of passive immunisation the 50% lethal dose (LD_{50}) was calculated according to the method of Reed & Muench (1938).

IMMUNOBLOTTING

SDS–PAGE separation of ECP from isolate 265-87-1 was carried out using the Mini-Protean II system from BioRad according to the manufacturers' instructions. The antigen was incubated for 5 min at 100°C with sample buffer (1:1 v/v) comprised of 8% SDS and 10% 2-mercaptoethanol in Tris–HCl, pH 6·8. Each gel was overlaid with 300 μl of antigen solution containing 200 μg protein. Molecular weight markers were BioRad, LMW. The stacking gel was 4·5% and the separation gel 14% acrylamide (Sigma, A7168).

Proteins separated by SDS–PAGE were transferred to a nitrocellulose membrane by electrophoresis at 100 V and 250 mA for 90 min. The buffer used for transfer was 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8·8. Following transfer, the membranes were either stained for total proteins with colloidal gold (AuroDye, Amersham) or blocked (1 h at 22°C) with 1% BSA, 1% normal swine serum in PBS-T. Primary and secondary antibodies, as well as the alkaline phosphatase-labelled IgG, were diluted in PBS-T containing 0·2% BSA and 0·2% swine serum. Incubation with primary antibody was at 4°C overnight, other antibodies were incubated for 1 h at room temperature. The blots were washed extensively in PBS-T between each step.

To determine the specificity of rabbit serum, immune or normal rabbit serum diluted 1/500 was used as the primary antibody. Bound antibody was detected with alkaline phosphatase-labelled swine antibody to rabbit IgG (Dako) diluted 1/2000.

To determine the specificity of salmon serum, the membranes were probed with immune or normal fish serum diluted 1/50, followed by incubation with a secondary polyclonal mouse antibody to salmonid IgM (raised against Atlantic salmon IgM at our laboratory) diluted 1/2000.

Bound antibody was detected with alkaline phosphatase-labelled rabbit antibody to mouse immunoglobulins (Dako). In order to inhibit non-specific binding, the conjugated antibody was incubated overnight at 4°C with normal salmon serum (10% v/v) before dilution.

Immunoreactive bands were visualised by incubating membranes in 0·1 M carbonate buffer pH 9·8 containing 0·3 mg/ml^{-1} p-nitro blue tetrazolium chloride (NBT) and 0·15 mg/ml^{-1} 5-bromo-4-chloro-3 indoxyl phosphate toluidine salt (BCIP).

III. Results

ACTIVE IMMUNISATION AND PROTECTION

Accumulated mortalities of actively immunised Atlantic salmon during the 28 day challenge are illustrated in Fig. 1. The challenge was effective with 86% mortality in the PBS control group. All four different vaccination schedules delayed the onset of disease and gave protection as compared with the PBS injected fish. No mortality of fish vaccinated with d-ECP occurred. Mortalities of fish vaccinated with d-ECP/f-Cells and f-Cells were 17 and 35%, respectively. In the adjuvant control group, 57% of the fish died from an *A. salmonicida* ssp. *achromogenes* infection.

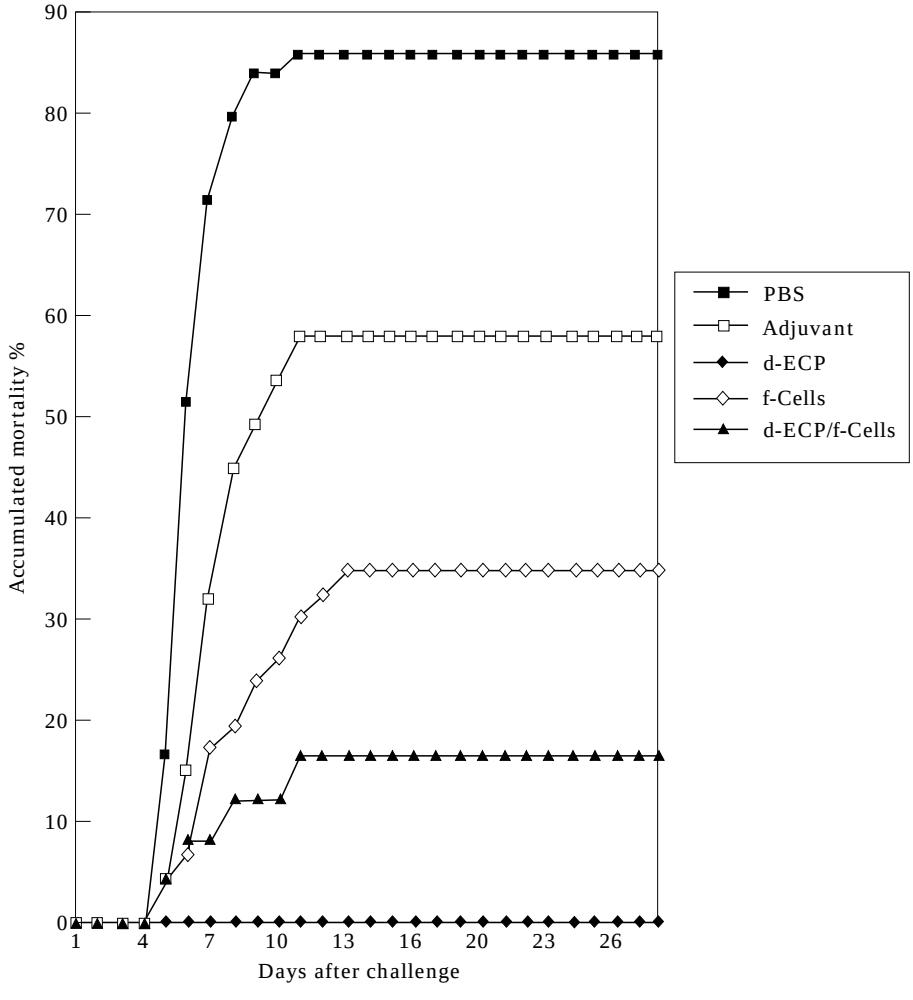

Fig. 1 Accumulated mortality (%) of Atlantic salmon following an i.m. challenge with *A. salmonicida* ssp. *achromogenes*, strain 265-87.

DETECTION OF ANTIBODIES IN SERA FROM ACTIVELY IMMUNISED ATLANTIC SALMON

Humoral antibody levels to ECP strain 265-87-1 (which secretes the A-layer protein) as measured by ELISA in sera collected at the day of challenge are listed in Table 2. Antibodies with binding capacity for ECP of strain 265-87-1 were detected in sera from fish in all test groups, except the PBS control group. Sera from fish vaccinated with both d-ECP and f-Cells had the highest ELISA value ($O.D._{492}$) or three times that of the adjuvant control sera. The anti-d-ECP sera had 50% and the anti-f-Cells sera 29% higher ELISA values than the adjuvant control sera. This indicates specific binding of the antigen. However, the adjuvant control sera had an approximately six times higher ELISA value than the PBS control, indicating that non-specific binding of the antigen was induced by the adjuvant.

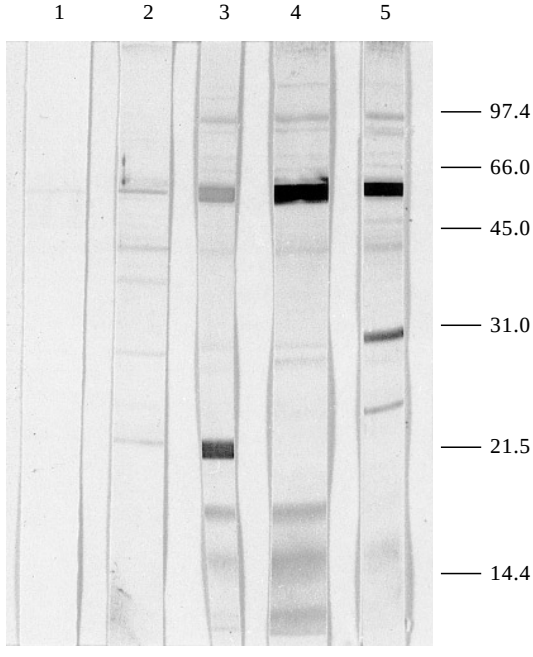

Fig. 2 Western blots of *A. salmonicida* ssp. *achromogenes* (strain 265-87-1) extracellular products (ECP). Proteins were detected by immunoblotting with sera (diluted 1:50) from Atlantic salmon injected with: Lane 1, PBS; Lane 2, PBS+adjuvant; Lane 3, d-ECP+adjuvant; Lane 4, f-Cells+adjuvant; Lane 5, d-ECP/f-Cells+adjuvant. Molecular weights are indicated in kDa.

Western blots of *A. salmonicida* ssp. *achromogenes* (strain 265-87-1) ECP probed with sera from vaccinated and control Atlantic salmon are shown in Fig. 2. No bands were visualised by PBS control sera (lane 1), but the adjuvant control sera stained several bands weakly, including a 50 kDa component (lane 2). All three sera pools from vaccinated fish detected a 50 kDa component and bands with molecular weights (MW) between 70 and 90 kDa and about 40 kDa (lanes 3, 4 and 5). A band with a MW of 20 kDa was only stained by sera from fish vaccinated with d-ECP (lane 3). Two bands with MW below 20 kDa were detected both by anti-d-ECP and anti-f-Cells sera (lanes 3 and 4). The sera from fish receiving vaccine containing both d-ECP and f-Cells stained two main bands with MW of 25 and 29 kDa, besides the bands stained by all three sera pools (lane 5). Probing of purified proteins with the same immune salmon sera showed that the 50 and 20 kDa bands were the A-layer protein and the extracellular metallo-caseinase, AsaP1, respectively (data not shown).

ANALYSIS OF ANTISERA USED FOR PASSIVE IMMUNISATION

The antibody titres of rabbit and rainbow trout control antiserum used for passive immunisation of Atlantic salmon tested by ELISA are listed in Table 3. The titres of the normal rabbit serum (NRS) and normal trout serum (NTS) used were 800 and 200, respectively, indicating some non-specific binding of

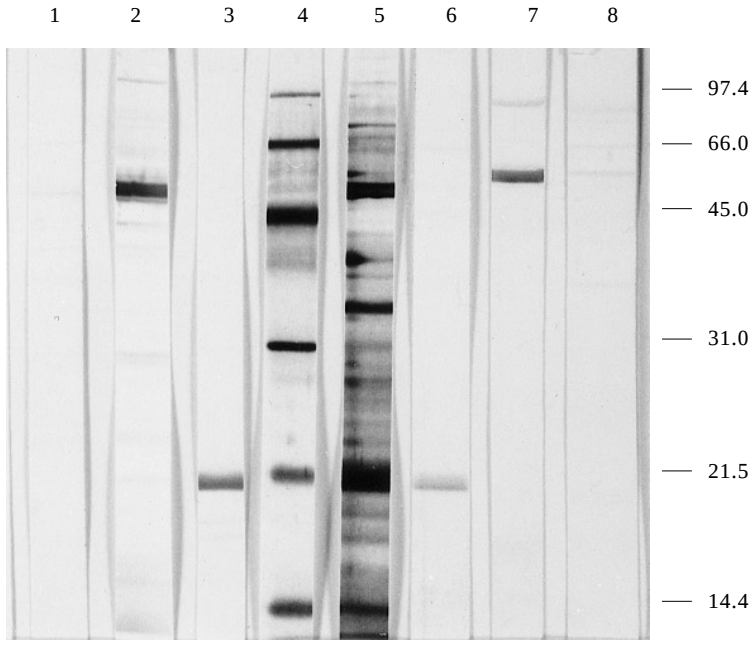

Fig. 3 Western blots of *A. salmonicida* ssp. *achromogenes* (strain 265-87-1) extracellular products (ECP). Proteins were detected with: Lane 1, normal rabbit serum (NRS); Lane 2, rabbit antiserum to the A-layer protein (Ranti-A-layer protein); Lane 3, rabbit antiserum to AsaP1 (Ranti-AsaP1); Lane 4, molecular weight standards (kDa) collodial gold stained; Lane 5, ECP proteins collodial gold stained; Lane 6, trout antiserum to AsaP1 (Tanti-AsaP1); Lane 7, trout antiserum to the A-layer protein (Tanti-A-protein); Lane 8, normal trout serum (NTS). The rabbit sera were diluted 1:500 and the rainbow trout sera pools (N=3) 1:50.

antigens in the test. The ELISA titres were higher in the rabbit than the trout sera and anti A-layer protein titres were higher than anti AsaP1 titres.

Western blotting analysis of control and immune rabbit and rainbow trout sera used for passive immunisation are shown in Fig. 3. Sera from the control animals showed no reactivity against the ECP of strain 265-87-1. Identical bands were detected with the rabbit and trout sera. The Ranti-AsaP1 and Tanti-AsaP1 sera stained a single 20 kDa band in the ECP but Ranti-A-layer protein and Tanti-A-layer protein stained a band with a molecular weight of 50 kDa strongly and also several others faintly.

PASSIVE IMMUNISATION AND PROTECTION

LD_{50} values were calculated for Atlantic salmon challenged 7 and 14 days after passive immunisation. Seven experimental groups were used in the study. Fish in two groups were passively immunised with rainbow trout serum and in another two groups with immune rabbit serum. Three control groups were included, fish in two of the control groups were treated either with normal trout or rabbit serum and one group of fish was injected with PBS instead of serum. The results are illustrated in Fig. 4. No significant difference in LD_{50} values was found in fish receiving Ranti-A-layer protein, Tanti-A-layer

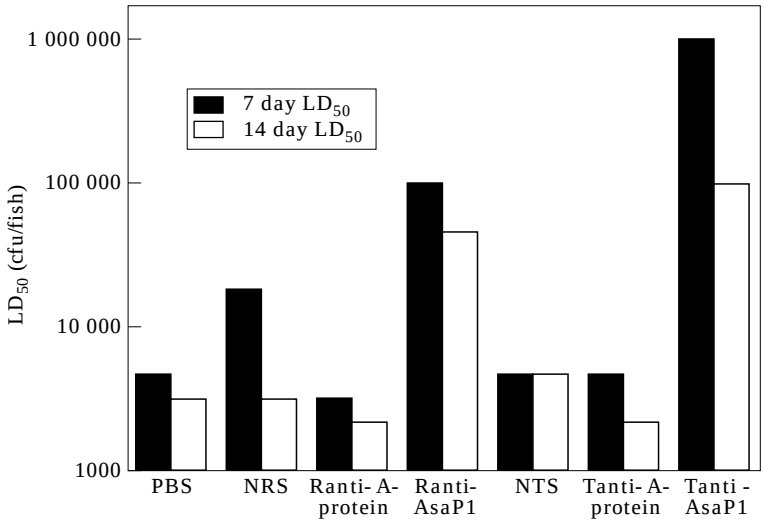

Fig. 4 LD_{50} values of Atlantic salmon calculated at 7 and 14 days post challenge with *A. salmonicida* ssp. *achromogenes*, strain 265-87. At the time of challenge groups of fish were injected with phosphate-buffered saline (PBS), normal rabbit serum (NRS), rabbit antiserum to the A-layer protein (Ranti-A-protein), rabbit antiserum to AsaP1 (Ranti-AsaP1), normal trout serum (NTS), trout antiserum to the A-layer protein (Tanti-A-protein) or trout antiserum to AsaP1 (Tanti-AsaP1).

protein, NRS, NTS or PBS, indicating that antibodies against the A-layer protein of *A. salmonicida* ssp. *achromogenes* did not confer passive protection against challenge. Control rabbit serum caused a four-fold increase in LD_{50} after 7 days compared with the PBS control group. This difference was not observed after 14 days. Control fish sera did not have any effect.

A considerable increase in LD_{50} was achieved in the two groups injected with serum containing antibodies to AsaP1. After 7 days, the Ranti-AsaP1 group showed a 22-fold increase in LD_{50} and the Tanti-AsaP1 group a 217-fold increase compared with PBS controls. If compared with the normal serum controls the respective values were the same for the Tanti-AsaP1 group and 7-fold for the Ranti-AsaP1 group. After 14 days, more mortalities had occurred in both groups and the same groups had increased LD_{50} values of 14-fold for the Ranti-AsaP1 group and 31-fold for the Tanti-AsaP1 group compared with PBS controls, but 14- and 22-fold, respectively, when compared with the normal serum groups. These results indicate that passive protection was achieved using both rabbit and trout AsaP1 antiserum and that considerably better protection was obtained when trout serum was used.

IV. Discussion

The results presented in this paper indicate that a specific humoral immune response evoked in Atlantic salmon by a 20 kDa extracellular metallocaseinase, AsaP1, plays an important role in protection against experimental infection of *A. salmonicida* ssp. *achromogenes*. Antibodies to the A-layer

protein were not protective to passively immunised Atlantic salmon. ECP were found to elicit better protection than whole bacteria in an experimental challenge of actively immunised Atlantic salmon.

In the present study, fish were actively immunised either with d-ECP, f-Cells or a mixture of both, in order to assess the protective role of cell-associated and extracellular antigens of *A. salmonicida* ssp. *achromogenes*. Freund's adjuvant (primary injection with FCA and booster of FIA) was introduced in the vaccine regimes used. A booster injection was given as it has been found to improve protection against atypical *A. salmonicida* infections in fish (Evenberg *et al.*, 1988; Daly *et al.*, 1994).

The results from the active immunisation experiment revealed that protection was achieved in all groups of vaccinated fish, all of which developed a specific humoral response directed against the A-layer protein and at least three other components. Fish injected with adjuvant alone also developed an immune response, which conferred some protection (Table 2). The best protection was achieved in fish vaccinated with preparations containing d-ECP. Thus, no mortality was detected in the group receiving d-ECP alone, but a 20% decrease in RPS value was observed when f-Cells were also included in this vaccine. Fish vaccinated with f-Cells had the lowest protection, with a RPS value 41% lower than that of the d-ECP group (Table 2). Therefore, ECP seem important in achieving protection against *A. salmonicida* ssp. *achromogenes*. There are no reports on protection of *A. salmonicida* ssp. *achromogenes* ECP in Atlantic salmon available, but our findings are in accordance with previous findings regarding infections of other *A. salmonicida* subspecies (Evenberg *et al.*, 1988; Arnesen *et al.*, 1993; Newman, 1993). The results also showed that all treatments, including the control injections of adjuvant, appeared to delay the onset of disease and gave some protection compared with the PBS-injected fish (Table 2). The stimulating effect of FCA in inducing protection to furunculosis in salmonids has been reported (Olivier *et al.*, 1986; Adams *et al.*, 1988).

As the vaccines were administered into the peritoneal cavity, the authors chose challenge by injection in the dorsal musculature in order to elude effects of a local immune response. This route of infection also produced clinical symptoms and disease propagation like those observed during natural infection. The challenge was effective for evaluation of vaccine-induced protection, with 86% mortality in the PBS control group. Protection against an injection challenge was not achieved in passive immunisation using either rabbit or rainbow trout sera containing antibodies against the A-layer protein of *A. salmonicida* ssp. *achromogenes*. Antibodies in sera from fish actively immunised with vaccines containing f-Cells were mainly specific against the A-layer protein, but the sera pool obtained from the d-ECP-vaccinated fish, which was better protected, bound the A-layer protein less effectively. This suggests that anti-A-layer protein antibodies were not of prime importance in protecting Atlantic salmon against an i.m. challenge of *A. salmonicida* ssp. *achromogenes*. However, the protection may be dependent on the route of challenge, as antibodies against A-layer protein have been shown to correlate with protection against furunculosis in Atlantic salmon challenged by cohabitation (Lund *et al.*, 1995).

Anti-AsaP1 antibodies were only detected in fish vaccinated with d-ECP, which all survived challenge. This indicates that induction of antibodies directed against the toxic metallo-caseinase, AsaP1, correlates strongly with protection of the salmon during experimental challenge. This was further supported by the observation that both monospecific rainbow trout and rabbit anti-AsaP1 sera, were able to protect Atlantic salmon against challenge with the virulent *A. salmonicida* ssp. *achromogenes* strain 265-87. Many reports suggest that the humoral immune response is not as critical as the cellular immune response in the development of immunity against *A. salmonicida* (Newman, 1993). However, the present study strongly indicates the importance of humoral antibodies to AsaP1 in the protection against *A. salmonicida* ssp. *achromogenes*.

Two extracellular components of 25 and 29 kDa were detected by anti-d-ECP/f-Cells antibodies but not with anti-f-Cells sera, and fish vaccinated with d-ECP/f-Cells had a higher survival following challenge. Therefore the 25 and 29 kDa components might also be antigens which induce protective antibodies. However, caution is necessary as regards conclusions based on antibody screening, because the samples used were serum pools taken at one time only.

The protection induced in Atlantic salmon was much better when trout rather than rabbit anti-AsaP1 sera were used for passive immunisation, although the ELISA titres were higher in rabbit than trout antisera and both sera were found to be monospecific by Western blotting. This could be explained by the difference of the immunoglobulins involved. Trout and salmon are closely related species composing tetrameric serum antibodies of only one class (IgM), but the rabbit serum antibodies would mainly be the monomeric IgG immunoglobulins.

In a previous study of Atlantic salmon naturally infected by *A. salmonicida* ssp. *achromogenes*, a strong response directed against the A-layer protein and LPS (O-antigen) in diseased fish was found. However, individual fish from a recently infected population were occasionally found to respond weakly against AsaP1 (Magnadóttir *et al.*, 1995). This is in agreement with our results as the response apparently had limited protective value and the disease progressed quickly unless controlled by other means, such as treatment with antibiotics.

The role of cell-mediated immunity in protection provided by active immunisation was not investigated in this study. In another recent study, where humoral antibody response also reflected challenge results, the effects of *A. salmonicida* ssp. *achromogenes* extracellular antigens on leucocyte cultures were studied in a blastogenic assay. The results showed that in addition to antibody-producing cells other cell populations were also proliferating (Gudmundsdóttir *et al.*, 1995). The results of the study presented here clearly demonstrate the importance of humoral antibodies and neutralisation of bacterial toxins in protecting Atlantic salmon against *A. salmonicida* ssp. *achromogenes*, although other immunological mechanism may also be involved.

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