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Evaluation of a whole cell, p57⁻ vaccine against *Renibacterium salmoninarum*

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ABSTRACT: A whole cell *Renibacterium salmoninarum* vaccine was developed using 37°C heat treated cells that were subsequently formalin fixed; this treatment reduced bacterial hydrophobicity and cell associated p57. Coho salmon *Oncorhynchus kisutch* were immunized with the p57⁻ vaccine by either a combination of intraperitoneal (ip) and intramuscular (im) injections or *per os*. In the first experiment, ip/im vaccination of coho salmon with p57⁻ cells in Freund's Incomplete Adjuvant (FIA) conferred a statistically significant increase in mean time to death after the salmon were ip challenged with 4.1×10^6 colony forming units (cfu) of *R. salmoninarum*. There was no significant difference in response between fish immunized with *R. salmoninarum* cell surface extract in FIA and those immunized with extracellular protein (ECP) concentrated from culture supernatant in FIA. The ip challenge dose resulted in complete mortality of all fish by Day 43. In a second experiment, fish were orally vaccinated with p57⁻ *R. salmoninarum* cells encased in a pH protected, enteric-coated antigen microsphere (ECAM). Fish were bath challenged with 4.2×10^6 cfu ml^{-1} on Day 0 and sampled at time points of 0 (pre-challenge), 50, 90, or 150 d immersion challenge. Vaccine efficacy was determined by monitoring the elaboration of p57 in the kidneys of vaccinated and control fish. Fish vaccinated orally demonstrated a significantly lower concentration of p57 ($p < 0.01$) at Day 150 post challenge compared to fish receiving ECAMs alone. Fish receiving p57⁻ cells without ECAM coating also showed a significantly lower p57 level ($p < 0.03$) versus control. In contrast, fish injected intraperitoneally with the p57⁻ cells or fish fed p57⁺ *R. salmoninarum* cells in ECAMs demonstrated no significant difference ($p > 0.05$) versus controls. In summary, these studies suggest the preliminary efficacy of 37°C treatment of *R. salmoninarum* cells as an oral bacterial kidney disease vaccine.

KEY WORDS: Bacterial kidney disease · *Renibacterium salmoninarum* · p57 · Oral vaccine

INTRODUCTION

The first reports of bacterial kidney disease (BKD) of salmon were made over 60 yr ago (Belding & Merrill 1935). To date, the disease remains one of the most problematic bacterial diseases of salmonids in the aquaculture industry. The responsible organism, *Renibacterium salmoninarum*, is a fastidious, slow growing, non-motile, Gram-positive, facultative intracellular parasite (Fryer & Sanders 1981) which causes a chronic and often fatal disease in a wide range of wild and cultured salmonid species (Austin & Austin 1987). The disease can cause severe losses among intensively cultured salmonids (Bullock & Herman 1988) and can be transmitted directly by the feeding of raw viscera of infected fish (Wood & Wallis 1955) or horizontally from infected fish sharing the same water supply (Mitchum & Sherman 1981, Bell et al. 1984). Reliance on diet modification, chemotherapy, and selective segregation of infected brood stock has been the only effective,

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albeit limited, means of controlling disease outbreaks (Elliott et al. 1989).

Prophylactic treatment through vaccination offers an ideal alternative for the control of BKD. However, investigators have had limited success at achieving a protective immune response against *Renibacterium salmoninarum* using standard methods for vaccine preparation (Patterson et al. 1981, McCarthy et al. 1984, Bruno 1988, Sakai et al. 1989, Evenden et al. 1993). Reasons for this limited success may be due to our general lack of knowledge of the mechanisms of pathogenesis and salmonid defense (Kaattari et al. 1988a,b). Also, failure to establish a reliable and natural challenge procedure has limited the success in this arena (Evenden et al. 1993).

Elimination of virulence factors from a prototype vaccine may benefit vaccine design. *Renibacterium salmoninarum* possesses a number of putative virulence factors that may contribute to its pathogenesis. Daly & Stevenson (1987) have reported on the ability of extracellular factors to agglutinate rabbit erythrocytes. Catalase, DNase, hemolytic, proteolytic and exotoxin activities have also been described (Bruno & Munro 1982, Shieh 1988, Evenden et al. 1993). Bruno (1988) found that isolates which do not auto-agglutinate have a reduced cell surface hydrophobicity and are less virulent. These isolates also lack a saline extractable 57 kDa protein (p57) (Bruno 1990). Work in our laboratory has identified an endogenous, PMSF sensitive, protease activity that can be enhanced by heat treating *R. salmoninarum* cells at 37°C for >10 h (Rockey et al. 1991, Wood & Kaattari 1996). This treatment results in reduced cell associated p57 and cell surface hydrophobicity (Piganelli et al. 1998, in this issue). We previously hypothesized that removal of p57 may allow a protective immune response (Kaattari et al. 1988b).

Until recently, lack of a natural challenge procedure has also impeded vaccine research (Elliott et al. 1991, Murray et al. 1992). Previous challenge procedures have utilized intraperitoneal (ip) injection of live cells (Bell et al. 1984); however, this method bypasses the skin and mucus, the first line of defense of the fish, and, therefore, is not relevant to natural exposure (Murray et al. 1992).

Alternatively, new challenge procedures consisting of bath challenges and prolonged incubation periods may more closely simulate natural exposure (Elliott et al. 1991, Murray et al. 1992). Also, protocols such as the soluble antigen enzyme-linked immunosorbent assays (ELISAs), which monitor the production of expressed antigen, can be used to detect the progression of the disease throughout the entire challenge period (Pascho & Mulchay 1987, Rockey et al. 1991). This latter procedure eliminates the necessity of solely noting the mean day to death for evaluation of vaccine efficacy.

In this manuscript, we describe the efficacy of the 37°C treated ($p57^-$) cell in affording protection upon ip as well as in bath challenge. The prototype vaccine was administered intraperitoneally as well as orally using enteric coated antigen microspheres (ECAMs).

MATERIALS AND METHODS

Salmon. Coho salmon *Oncorhynchus kisutch* were obtained from the Oregon Department of Fish and Wildlife hatchery at Sandy, Oregon. Fish were kept at the Salmon Disease Laboratory, Oregon State University, in 12°C water. Fish were fed Oregon Moist Pellet (OMP) (Bioproducts, Astoria, OR) daily. The fish each weighed 45 g at the start of the experiment.

Preparation of bacterial cells and ECP. *Renibacterium salmoninarum* isolate D6 (originally obtained from C. Banner, Oregon Department of Fish and Wildlife, Oregon State University, Corvallis, OR) was grown in seven 1 l volumes in 2.5 l low form, culture flasks (VWR) with continuous shaking at 17°C; KDM-II medium prepared according to Evelyn (1977) except without serum supplementation was used. Bacteria were grown for 7 to 8 d until an absorbance between 0.4 and 0.8 (525 nm) was generated. Cells were pelleted by 6000 × *g* centrifugation for 30 min and resuspended in 100 ml cold phosphate buffered saline (PBS; 0.85% NaCl, 10 mM $NaPO_4$, pH 7.2). After a second centrifugation, the cells were placed in microfuge tubes and frozen at −70°C for further use. Extracellular protein (ECP) was extracted from culture supernatants as previously described (Wiens & Kaattari 1989).

Preparation of bacterial cell surface extract (CSE). A CSE was prepared according to the method of Daly & Stevenson (1990). Briefly, 2 to 4 g wet weight of bacterial cells was washed with 100 ml of sterile PBS and pelleted at 6000 × *g* for 30 min. Cells were resuspended in 100 ml of double distilled water for 1 h on ice and the cells were repelleted, the supernatant removed and precipitated with the addition of powdered ammonium sulfate to yield a final 50% weight/volume solution. The CSE was dialyzed 3× against PBS overnight and filter sterilized (0.45 µm, Gelman Scientific). Protein concentration was determined by the method of Lowry et al. (1951).

Vaccine preparation. Previously harvested cells were thawed from −70°C, microfuged for 2 min (750 × *g*), weighed and resuspended to 200 mg ml^{-1} in sterile, cold PBS. Cells were placed at 37°C for 48 h. Upon completion of incubation, cells were microfuged and resuspended into 3% formalin PBS at 17°C for 10 h. Cells were washed 3× with 1 ml PBS and re-weighed.

Antigen preparation for the intraperitoneal/intramuscular vaccination and challenge experiment. Antigens for the ip/im immunization and challenge experiment were prepared as follows: All antigens tested as well as 1 PBS control were emulsified in Freund's Incomplete Adjuvant (FIA) for 4 min at 100 units on a Virtis '23' mixer (Virtis Co., Gardiner, NY). PBS and PBS/FIA alone served as controls. The emulsified antigens were as follows: 37°C heat treated p57⁻ *Renibacterium salmoninarum* cells (500 µg), CSE (50 µg) from water washed whole cells, and ECP (50 µg) concentrated from culture supernatants as described previously (Wiens & Kaattari 1989). Injections were given intraperitoneally and intramuscularly, in a total volume of 0.1 ml, delivering 0.05 ml in each site using a 23 gauge needle. Boosts were followed 45 d after primary injection and consisted of one half the quantities used in the primary injection contained in the same total volume. A secondary boost was given 10 d after the first boost; the concentrations of the antigens remained the same as in the first boost. Triplicate tanks containing 45 fish $tank^{-1}$ were used to test the 3 vaccine treatments and 2 controls.

Intraperitoneal challenge. *Renibacterium salmoninarum* used in the challenge was grown for 7 d in KDM-II and washed 1× in PBS. Cells were resuspended in sterile PBS to a final absorbance of 0.2 (525 nm) and delivered intraperitoneally to the fish. Plate counts confirmed the challenge dose to be 4.1×10^6 colony forming units (cfu) ml^{-1}. Mortalities were recorded daily. Gram staining and indirect immunofluorescent assay (IFA) (Bullock et al. 1980) were used to confirm the presence of *Renibacterium salmoninarum* in a portion of the mortalities (data not shown).

Oral vaccine preparation. Previously harvested cells were thawed for use in preparing the respective antigens for oral vaccination. The antigens for the oral ECAM experiment were as follows: ECAMs were prepared by spray-coating the antigen, either 37°C treated (oral p57⁻) or non-heat treated cells (oral p57⁺), onto 40–50 mesh dextrose beads (Paulaur Corp., Princeton Junction, NJ) at a dose of 100 µg antigen $fish^{-1}$ d^{-1}. The 100 µg of antigen $fish^{-1}$ d^{-1} was calculated as follows: Based upon a food consumption of 7 g d^{-1} for the 25 fish in each tank, 1.5 g of beads (40–50 mesh dextrose beads), containing 2.5 mg of antigen, was incorporated into 7 g of fish diet. Therefore, the 2.5 mg of antigen given to the 25 fish in each tank is equal to 100 µg antigen $fish^{-1}$ d^{-1}. The beads were then spray-coated with Eudragit L-30D, a commercially available aqueous, pH-reactive latex dispersion of methacrylic-acrylic acid co-polymer. The 37°C treated p57⁻ cells were also coated and administered at a 100 µg $fish^{-1}$ d^{-1} dosage without the pH-reactive polymer to serve as a non-pH protected control (oral NPP p57⁻). The final control consisted of non-antigen coated, pH protected ECAMs alone (control). The ECAMs were mixed uniformly throughout the mash of Oregon Moist Pellet (BioProducts) and distilled water was added to form a mull. The mixture was then extruded through a defined pore size extruder (Vitano, EastLake, OH) and was cut into pellets. The fish were fed the respective ECAMs incorporated in their diet. The fish received ECAM incorporated feed on an every other day basis for a total of 30 d. An ip injection treatment was incorporated in the oral vaccine study and consisted of 37°C heat treated p57⁻ emulsified in FIA (ip p57⁻) as described, with modifications to injection. Fish received 500 µg injected in a volume of 0.1 ml anterior to the pelvic fin using a 26 gauge needle. The boost schedule for the ip group followed the same schedule as the previously described ip injection experiment. After ECAM and ip injection schedules were complete the fish were rested 20 d and then challenged.

Triplicate tanks containing 25 fish $tank^{-1}$ were used to test the 4 vaccine treatments and the 1 control. To monitor the humoral responses and pre-challenge soluble antigen titers, 5 fish $tank^{-1}$ were sacrificed. Sera and kidney samples were collected from 5 fish $tank^{-1}$ prior to challenge, in order to monitor pre-humoral and soluble antigen titers.

Bath challenge for oral vaccine experiment. *Renibacterium salmoninarum* (D6 strain) was grown as previously described. The growth from three 1 l flasks was combined. Fish were exposed to *R. salmoninarum* by bath challenge as described by Elliot et al. (1991) with modifications. The water level in each tank was first reduced from 125 to 25 l. The flow of water was then stopped, and supplemental aeration initiated. Viable *R. salmoninarum* was added to the tanks to give a final concentration of 4.2×10^6 cfu ml^{-1} as determined by plate count. The fish were exposed to the bacteria for 22 h in the standing aerated water. Following the 22 h exposure the water flow was resumed and the tanks were allowed to fill at a rate of 2.8 l min^{-1}, thus removing bacteria through normal effluent flow. One replicate tank of the non-heat treated (oral p57⁺) vaccinated fish was lost due to interruption of air to the tank.

Determination of antibody activity. Antibody activity titers were ascertained by the use of an ELISA as previously described (Piganelli et al. 1994, Wood & Kaattari 1996) with modifications. Briefly, each antiserum was titrated on an ELISA plate (Costar E.I.A/R. I. A. certified surface chemistry, Cambridge, MA) using formalin fixed non-heat treated *Renibacterium salmoninarum* as the coating antigen (150 µg ml^{-1}). Each plate contained a titration of an anti-*R. salmoninarum* hyperimmune serum generated in coho salmon.

This latter titration permits normalization of the data and a standardized estimation of the units of activity per ml serum (Arkoosh & Kaattari 1990).

ELISA-based monitoring of disease progression. Monitoring the progress of infection after challenge was accomplished using the monoclonal antibody-based ELISA protocol described by Rockey et al. (1991), with modifications. The ELISA quantifies the amount of p57, a major component of the ECPs produced by *Renibacterium salmoninarum*. Briefly, 5 fish from each triplicate treatment were euthanized in order to monitor levels of soluble antigen. Kidney samples were taken at 0 (pre-challenge), 50, 90 and 150 d post challenge. Samples were collected from fish using fresh collection tools for each individual to minimize cross-contamination. Samples were placed into microfuge tubes held on ice and then mixed 1:1 (weight:volume) with cold 1% bovine serum albumin in Tween 20-Tris buffered saline (BSA/TTBS) (50 mM Tris, 1 mM EDTA, 8.7% NaCl and 0.1% Tween 20 [pH 8.0]). The samples were then homogenized via repeated passage through a 1 ml syringe. Supernatants were collected as described by Rockey et al. (1991). ELISAs were performed on all samples according to the protocol of Rockey et al. (1991), with modifications. Standard concentrations of p57 were run on every plate in order to generate a standard curve. Incubation times were followed as described by Rockey et al. (1991), and absorbance at 405 nm was measured on a Titertek Multiscan Plus plate reader (Flow Laboratories). Derivation and analysis of the linear equation from the average of triplicate wells for each sample as well as the standard curve were conducted using the DeltaSoft ELISA program software package (BioMetallics). The concentration of p57 in each sample was calculated as described by Rockey et al. (1991), using absorbance values generated from the standard curve. The assay has a baseline detection limit of 3 ng ml^{-1}

Polyacrylamide gel electrophoresis and western blotting. Polyacrylamide gel electrophoresis and western blotting were performed as previously described (Wiens & Kaattari 1989). Preparations of *Renibacterium salmoninarum* cells used as antigens in the vaccine experiments were electrophoretically analyzed as described in Wiens & Kaattari (1991).

Statistical analysis. Due to the variance in p57 levels among individual fish, all data were log transformed. Data were analyzed using the 1-way analysis of variance (ANOVA) program on the Statgraphics software package. ANOVA generates an *F*-statistic which is subsequently analyzed by the *t*-test to determine significance using the pooled standard deviation (SP2). The 99 and 95% confidence limits were derived using standard error of the mean.

RESULTS

Total protein and western blot analysis of whole *Renibacterium salmoninarum* cell vaccine preparations

The removal of p57 after 48 h incubation at 37°C followed by formalin treatment was assessed by both total protein (Fig. 1A) and western blot (Fig. 1B). Electrophoresis of equal amounts (50 µg) of 37°C and 4°C treated cells revealed a strong p57 band in the 4°C cell lysates but not in the 37°C samples.

Effect of ip/im vaccination and challenge

The first vaccine experiment compared the effect of *Renibacterium salmoninarum* CSE, ECP and 37°C $p57^-$ whole cells. Each fish was challenged ip with 4.1×10^6 cfu of live *R. salmoninarum* cells. The challenge dose resulted in complete mortality in all tanks by 43 d. No difference in mortality was observed in mean time to death between CSE and ECP vaccine treatments and the saline or saline/FIA controls, (Fig. 2A–C). However a significant extension of the mean time to death was observed ($p < 0.05$) in the $p57^-$ whole cell vaccine treatment (Fig. 2D).

Effect of oral (ECAM) or ip vaccination and bath challenge

Fish which were vaccinated by either ECAM (oral) delivery or ip injection were subsequently bath chal-

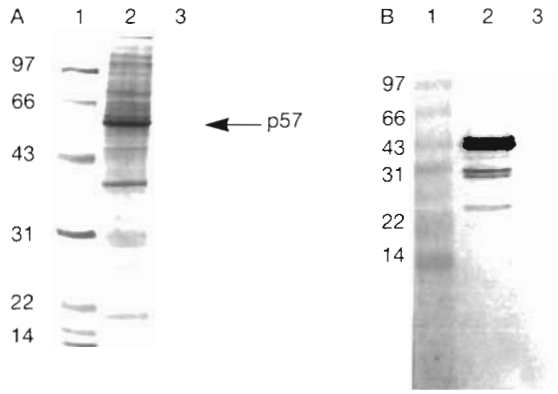

Fig. 1. *Renibacterium salmoninarum*. (A) Total protein stain and (B) western blot after 37°C treatment for 48 h followed by 0.3% formalin incubation at 17°C for 10 h. Lanes: (1) molecular weight markers, (2) untreated *R. salmoninarum* cells, (3) 37°C treated *R. salmoninarum* cells. Western blot was probed for p57 using monoclonal antibody 4D3 (Wiens & Kaattari 1989)

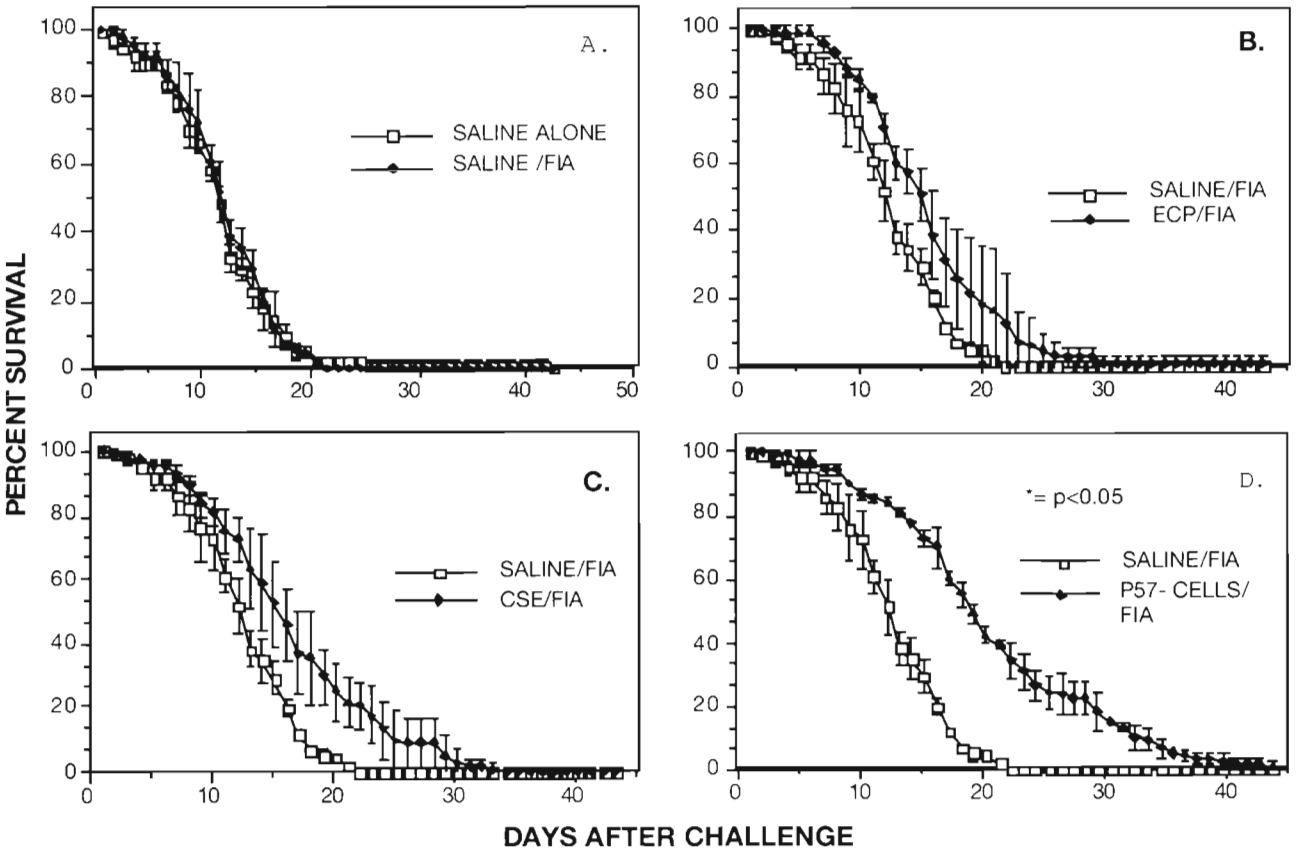

Fig. 2. *Oncorhynchus kisutch*. Percent survival of coho salmon immunized with (A) saline/FIA, (B) ECP/FIA, (C) CSE/FIA and (D) p57⁻ cells/FIA. For simplicity each treatment was graphed against the FIA control. Three tanks of 45 fish were used for each treatment. Mean percent survival and standard deviation were calculated for each day after challenge with 4.1×10^6 cfu. *Significant difference at the 0.05 level ($p < 0.05$)

lenged. Progress of the infection was monitored over time by the determination of p57 production. There were no significant differences between treatments versus non-antigen coated control beads (control) at 0 (pre-challenge), 50 and 90 d post challenge (Table 1); however, by 150 d, significant differences were observed. Fish fed the oral p57⁻ cell vaccine contained significantly less p57 in kidney tissue compared to the controls ($p < 0.01$) (Table 1). In addition, the oral NPP p57⁻ vaccinated fish contained significantly less p57 in kidney tissue versus controls ($p < 0.03$). In contrast, at Day 150, the concentration of p57 in the ip p57⁻ and the oral p57⁺ vaccinated groups was not significantly different from the control, ECAM vaccinated group ($p > 0.05$).

Table 1. *Renibacterium salmoninarum*. Mean p57 production (ng ml^{-1}) detected after ECAM (oral) or ip vaccination for each particular treatment on each sampling day in the bath challenge. Standard errors are given in parentheses. **$p < 0.01$, *$p < 0.05$ versus control

Treatment	Number of fish sampled per time point	Pre-challenge	50 d post challenge	90 d post challenge	150 d post challenge
Control[a]	15	<3[f]	3	351 (352)	2070 (1600)
Oral p57⁻[b]	15	<3	<3	20 (18)	<3**
NPP p57⁻[c]	15	<3	<3	21 (17)	3*
Oral p57⁺[d]	10	<3	<3	8701 (8600)	8403 (5603)
ip p57⁻[e]	15	<3	<3	12900 (6400)	220 (173)

[a]Control non-antigen coated beads alone; [b]ECAM delivered p57⁻ whole cells; [c]Non-pH protected p57⁻ whole cells; [d]ECAM delivered p57⁺ whole cells; [e]Intraperitoneal injected p57⁻ whole cells; [f]Detection limit of assay

Table 2. *Renibacterium salmoninarum*. Mean number of serum antibody units of activity per µl serum detected after ECAM (oral) or ip vaccination for each particular treatment on each sampling day in the bath treatment. Standard errors are given in parentheses. nd = not detectable

Treatment	Number of fish sampled per time point	Pre-challenge	50 d post challenge	90 d post challenge	150 d post challenge
Control[a]	15	nd	nd	379 (272)	2060 (2276)
Oral p57$^-$[b]	15	nd	nd	nd	126 (106)
NPP p57$^-$[c]	15	nd	nd	938 (570)	1827 (738)
Oral p57$^+$[d]	10	nd	nd	500 (320)	3423 (603)
ip p57$^-$[e]	15	5800 (748)	42400 (13432)	82000 (56312)	14776 (6119)

[a]Control non-antigen coated beads; [b]ECAM delivered p57$^-$ whole cells; [c]Non-pH protected p57$^-$ whole cells; [d]ECAM delivered p57$^+$ whole cells; [e]Intraperitoneal injected p57$^-$ whole cells

Serum antibody titers were monitored from each fish and the titers indicate that the ip p57$^-$ treatment elicited specific serum antibodies to formalin fixed *Renibacterium salmoninarum* cells throughout the sample period (Table 2). The antibody titers in the ip p57$^-$ group were much greater than found in the ECAM vaccinated groups (Table 2) and did not correlate with protection as indicated by the high p57 levels in kidney tissue.

DISCUSSION

We examined the efficacy of a vaccine based on the heat treatment of *Renibacterium salmoninarum* cells administered intraperitoneally by injection as well as orally using ECAMs. The first experiment employed ip injection with an ip challenge, and demonstrated that a significant increase in mean day to time to death ($p < 0.05$) was conferred by p57$^-$ in FIA whole cell immunization. There was no significant difference between fish immunized with CSE or ECP in FIA compared to the saline or saline/FIA controls, suggesting that components present in or on *R. salmoninarum* cells may be necessary to elicit protective immunity. While we observed a significant delay in the mean day to death in the p57$^-$ vaccine group, the ip exposure chosen for challenge eventually resulted in complete mortality for all fish. The ip route of challenge is problematic as it bypasses the outer integument of the fish and introduces *R. salmoninarum* in an unnatural way. In fish, unlike mammals, the entire interface with the environment consists of a mucosal epithelium, therefore it would seem that these mucosal surfaces play a critical role in the fish's first line defense (Davidson et al. 1993). Therefore, a second experiment was conducted in order to assess vaccine efficacy, utilizing a bath challenge as it is a more natural route of pathogen exposure. Challenge by bath immersion has been established to be an effective model for studying *Renibacterium salmoninarum* infection in a laboratory setting (Murray et al. 1992). The disadvantage of the bath challenge method is that considerable time is needed to reach significant mortality. This disadvantage has been circumvented with the advent of diagnostic ELISAs that monitor the production of antigens by *R. salmoninarum in situ*. ELISA analysis of antigen levels allows a more rapid analysis of the progression of infection and provides a means of quantifying vaccine efficacy (Elliott et al. 1991).

Fish in the second experiment were vaccinated by ECAM feeding or by conventional ip injection. All fish were then bath challenged. The results of this experiment demonstrated that route of administration was important when natural challenge conditions were employed. Fish fed p57$^-$ whole cells in ECAMs (oral p57$^-$) contained significantly lower amounts of p57 in kidney tissues ($p < 0.01$) at 150 d post challenge as compared to control fish. The increase in p57 concentrations in the kidney of control fish is indicative of *Renibacterium salmoninarum* infection. Fish fed unprotected p57$^-$ cell coated beads (oral NPP p57$^-$, without the pH polymer protection) also showed a significant decrease ($p < 0.03$) in p57 concentration versus control; however, the significance was not as great as with the pH protected ECAMs. The fish fed ECAMs coated with the non-heat treated cells (oral p57$^+$) showed no statistical difference versus controls with respect to p57 levels throughout the entire experiment. Fish receiving an ip injection of p57$^-$ cells (ip p57$^-$) demonstrated measurable and, on the average, higher units of anti-*R. salmoninarum* serum antibody throughout the entire experiment, yet p57 amounts were not different from controls.

When the antibody titers were examined, it became apparent that the fish with the most severe disease or highest concentration of p57 (ip p57$^-$) also possessed the greatest antibody response. Interestingly, groups exhibiting the lowest level of p57 (oral p57$^-$ and NPP p57$^-$) possessed the lowest antibody titers. Thus, the

appearance of serum antibody titers in these experiments is contraindicative of protection. These results are consistent with other reports which relate that fish exposed to *Renibacterium salmoninarum* can mount an immune response as indicated by antibody titers; however, the presence of this has not correlated with protection against the disease (Evelyn 1971, Patterson et al. 1981, Bruno 1987). Recently it has been reported by Davidson et al. (1993) that route of immunization has an impact on the generation of antibody secreting cells in the gut of rainbow trout *Oncorhynchus mykiss*. They demonstrated that oral immunization produces a faster peak antibody titer (3 wk) in both the intestinal mucosa and head kidney as compared to ip injection (7 wk). The rapid induction of a peak antibody titer in the intestinal mucosa may explain the efficacy of the oral p57⁻ vaccine. A mucosal response may have allowed for protection at the mucosal epithelium, which is crucial for disease resistance when the route of exposure is through mucosal sites (Finlay & Falkow 1989, McGhee et al. 1991). Lobb (1986) demonstrated that bath immunization of channel catfish *Ictalurus punctatus* produced antibody in cutaneous mucus in 5 of 6 individuals following immersion in dinitrophenylated horse serum albumin (DNP-HSA). However, only 1 out of 5 fish demonstrated a marked increase in serum anti-DNP antibody following the bath immersion. Lobb (1986) also demonstrated that the channel catfish possess numerous lymphocytes associated with the epidermis. This study suggests that the channel catfish immune system can respond differentially depending on the route of exposure. Other reports have demonstrated that serum antibody titers of bath or orally immunized fish do not correlate with protection against certain pathogens. Croy & Amend (1977) observed significant protection with bath immunization against vibriosis in the absence of serum titers. Because *R. salmoninarum* is a facultative intracellular parasite, the role of protection by cellular immunity should not be overlooked. However, more work on and development of cell mediated assays are needed to explore this possibility. Recently, Wood & Kaattari (1996) demonstrated that removal of p57⁻ from the *R. salmoninarum* cell surface by activation of endogenous serine protease resulted in a 20-fold increase in the detectable antibody titer in chinook salmon *Oncorhynchus tshawytscha* versus the response to intact p57⁺ *R. salmoninarum* cells. Further treatment of the p57⁻ cells with periodate revealed that the increased antibody response was almost exclusively to unmasked carbohydrate moieties. Clearly, if oral delivery of the ECAMs coated with p57⁻ cells (oral p57⁻) elicited a mucosal immune response to these unmasked carbohydrate antigens, then protection may have been achieved at the initial site of entry. However, we cannot rule out the possibility that other proteins may have also been removed during the heat treatment of the *R. salmoninarum* cells, which may have contributed to the protection seen with the oral p57⁻ vaccine. It is tempting to speculate that the protection seen in the oral p57⁻ group was due to the removal of the p57 protein, which may have altered the antigenic characteristics recognized by the salmonid immune system.

In conclusion, the results presented here demonstrate that heat treatment of *Renibacterium salmoninarum* cells and route of delivery are critical factors to consider if protection against *R. salmoninarum* infection is to be achieved. The orally delivered p57⁻ cells (oral p57⁻) afforded significant protection when fish were exposed to viable *R. salmoninarum* by bath immersion, while the ip p57⁻ and oral p57⁺ cells did not. Although in the first experiment the ip p57⁻ vaccine demonstrated a significant delay in mean time to death after ip challenge, the results of the second experiment proved that when salmon were challenged by a natural route this method of immunization was significantly less effective. These results show promise for elucidating the ideal candidate antigen(s) and developing oral vaccines for the stimulation of a protective immune response in salmonids against BKD.

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