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Protective immunity in juvenile coho salmon *Oncorhynchus kisutch* following immunization with *Vibrio ordalii* lipopolysaccharide or from exposure to live *V. ordalii* cells

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ABSTRACT: Naive, juvenile coho salmon *Oncorhynchus kisutch* were protected against an experimental challenge with the fish pathogen *Vibrio ordalii* following vaccination with *V. ordalii* lipopolysaccharide (LPS) administered by either the intraperitoneal or immersion route. Purified LPS extracted from *V. ordalii* cell walls (CW-LPS) and semi-purified extracellular LPS from the broth used for growing *V. ordalii* cells (EC-LPS) resulted in protection by each of these routes. Amounts of CW-LPS as low as 100 pg via the intraperitoneal route and 500 ng ml^{-1} via the immersion route were effective in protecting 4.8 g coho salmon against *V. ordalii*. Naive, juvenile coho salmon surviving bath exposure to live cells of *V. ordalii* also developed immunity to the pathogen. Exposure for 15 min to numbers of *V. ordalii* cells low enough not to cause vibriosis (2.8×10^1 to 1.1×10^6) resulted in measurable immunity to *V. ordalii*. These results are consistent with those obtained in this laboratory and elsewhere with the related but better studied fish pathogen *V. anguillarum*. We also document in quantitative terms, and for the first time, the extremely high level of immunogenicity associated with *V. ordalii* LPS.

INTRODUCTION

Vibrio vaccines have been used with considerable success in various parts of the world to protect salmonids farmed in seawater against losses due to vibriosis (Evelyn 1984, 1988). In British Columbia (B. C.), Canada, bacterins containing the 2 fish pathogenic vibrios indigenous to the region's coastal waters (*Vibrio anguillarum* = *Va*, and *Vibrio ordalii* = *Vo*) have routinely been used for this purpose. Early work with vibrio bacterins in B. C. indicated that their immunogenicity was due to a substance occurring in both the free and cell-associated form (Evelyn & Ketcheson 1980). Initial evidence suggested that the immunogens were lipopolysaccharides (LPS): they were large, heat-stable molecules that were resistant to the harsh techniques used for their extraction and purification (reviewed by Evelyn 1984). Further, LPS was known to be a major surface antigen of *Va* and *Vo* (Chart & Trust 1984). Direct evidence that LPS was indeed the immunogen was obtained when purified LPS of *Va* (Kawai & Kusuda 1983, Aoki et al. 1984, Salati et al. 1989) and of *Vo* (Evelyn & Wong cited in Evelyn 1988) was successfully used to immunize salmonids against experimental challenge with *Va* and *Vo*, respectively.

In this study we focussed our attention on the less well studied *Vo*. Our purpose was, first, to determine how much *Vo* LPS was required to induce immunity in juvenile salmon when administered by intraperitoneal and immersion routes. This goal has important implications regarding the cost of vaccinating salmonids against vibriosis.

Our second goal was to determine whether exposure to live *Vo* cells would serve to immunize young salmon against subsequent challenges with the pathogen. Exposure to live *Va* cells had already been found to protect young salmonids against *Va* (Braaten & Hodgins 1976, Egidius & Andersen 1979, Evelyn & Ketcheson 1980, Thorburn et al. 1987).

MATERIALS AND METHODS

Three experiments were conducted. The first 2 examined the efficacy of vaccinating juvenile coho salmon *Oncorhynchus kisutch* with various doses of *Vo*

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LPS administered by each of 2 methods: by intraperitoneal injection (IP) and by the immersion method (IMM). The third experiment examined the effect of exposing juvenile coho salmon to various concentrations of live *Vo* cells and the subsequent ability of the fish to resist an experimental challenge with live *Vo*.

Bacterium. The *Vo* isolate (# 74/48) used in this study was obtained from a seawater-reared sockeye salmon *Oncorhynchus nerka* that had died of vibriosis in 1974 (Evelyn & Ketcheson 1980). The isolate was typical of *Vo* and has been described elsewhere (Schiewe et al. 1981).

LPS preparation and quantitation. When used as a source of cell wall-associated LPS (CW-LPS) or of extracellular LPS (EC-LPS), the isolate was grown at 22 °C for 32 h in aerated Brain Heart Infusion Broth (Difco), supplemented with 1 % NaCl. Growth was stopped by the addition of formalin to a final concentration of 0.5 % (v/v) and the formalin-treated culture was left overnight with stirring. The broth culture was then processed as follows to obtain the CW-LPS and EC-LPS. *Vo* cells were pelleted by centrifugation at 10 400 × *g* for 30 min at 4 °C and washed twice with phosphate buffered saline (PBS = 0.15 M, pH 7.4). CW-LPS was extracted from the cells by the method of Darveau & Hancock (1983). The spent broth was centrifuged again and the supernatant collected. The supernatant was then diluted 1:2 with ultrafiltered (YM100 filter, Amicon Corp.) PBS and concentrated over a YM100 filter using a 200 ml positive-pressure cell (Amicon Corp.). The retentate was washed thoroughly with PBS until the absorbance of the filtrate at 280 nm was the same as that of PBS. The retentate was then centrifuged at 50 000 × *g* for 30 min to pellet any fine particles present. The supernatant was then ultracentrifuged at 200 000 × *g* for 2 h to pellet the semi-purified EC-LPS and then washed twice with Tris buffer (0.01 M, pH 8.0). Both types of LPS were quantitated by the chromogenic limulus amoebocyte lysate method (M. A. Whittaker, Bio-products). The CW-LPS and EC-LPS were also characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli 1970), and by silver staining using the method of Tsai & Frasch (1982) as modified by Hitchcock & Brown (1983). These preparations were found to have silver-stained profiles similar to the cell wall LPS extract of *Vo* described by Chart and Trust (1984). We detected protein in the EC-LPS, but not in the CW-LPS, when the gels were loaded with 5 times the amount of material required for detecting LPS and were then stained with Coomasie blue. Protein levels in our LPS preparations were quantified using the Bradford-based protein assay (Bio-Rad). There was 1.2 % protein in the CW-LPS and 2.6 % protein in the EC-LPS.

Challenge suspensions. When the isolate was used as a live vaccine (Expt 3) or for challenging the various groups of vaccinated and control fish, it was grown for 42 h at 22 °C on Tryptic Soy Agar (TSA; Difco), supplemented with 1 % NaCl. The cells were then harvested and suspended in peptone (0.1 %)-saline (0.9 % NaCl) (PS). They were then diluted to a standard turbidity of 1.0 at 540 nm, and further dilutions of this suspension were made in PS to obtain challenge suspensions of the desired strength and to permit counts of the existing viable cell population. The technique of Miles & Misra (1938) was used for the latter purpose.

Fish and fish handling. The coho salmon used in the study were under-yearlings and averaged 4.8 g and 13.5 g, depending on the experiment in which they were used. They were obtained from a local (the Big Qualicum River) stock and were reared in well water at the Department of Fisheries and Oceans Rosewall Creek facility, some 80 km north of our laboratory. The fish were transported to our laboratory at the Pacific Biological Station at least 10 d prior to use and were held in an 800 l tank, supplied with flowing dechlorinated city water. During this period, fish were gradually acclimatized to a temperature of 13 ± 1 °C.

Vaccination procedures. In Expts 1 and 2, fish vaccinated by the IP route were first anesthetized with 2-phenoxyethanol (0.037 % v/v) to facilitate their handling and minimize stress. They were then injected with 0.1 ml of sterile, pyrogen-free water (controls) or with 0.1 ml of sterile, pyrogen-free water containing various test doses of LPS (see Tables 1 and 2 of the 'Results and Discussion' section for details). Fish immunized by immersion in Expts 1 and 2 were not anesthetized because anesthesia had been shown to reduce the uptake of a number of antigens (Evelyn 1984). These fish were immersed in 2.0 l of aerated fish-culture-water (controls) or in 2.0 l of aerated fish-culture-water containing the desired concentrations of LPS. Immersion was carried out at 13 °C for 3 or 30 min. In Expt 3, fish were vaccinated without anesthesia by immersing them for 15 min in 2.0 l of aerated PS (controls) or in 2.0 l of aerated PS containing the desired concentrations of viable *Vo* cells at 13 °C (see Table 3 of the Results and Discussion section for details). Two 25-fish lots of coho salmon were used for each treatment, except where otherwise specified, and the lots were held in separate 35 l tanks, each of which was supplied with flowing dechlorinated city water at 13 ± 1 °C. Following vaccination, fish were held for 21 d (Expts 1 and 2) or for 13 to 21 d (Expt 3) at 12 to 14 °C and then challenged.

Challenge method. Challenge consisted of an IP injection containing 0.1 ml of the *Vo* cell suspension, described above. Challenged fish were monitored for mortalities for 14 d, by which stage all mortalities

appeared to have ceased. Kidney samples from 10 % of the dead fish were cultured on TSA containing 1 % added NaCl to verify that *Vo* was the cause of death. Identification of *Vo* growth on the plates was accomplished serologically (agglutination test) using a *Vo*-specific rabbit serum (Gibco).

Agglutination titers. Blood samples were collected from the severed caudal peduncles of anesthetized fish in heparinized Caraway tubes. Five fish were sampled prior to vaccination, and 10 fish per treatment (5 fish per tank) were sampled just prior to challenge. The blood was centrifuged and the resulting plasma samples titrated for their *Vo* agglutinin levels using routine procedures in microtiter plates and formalin-killed *Vo* cells.

Statistical analysis. Mortalities in the various replicate groups of fish were pooled (to give deaths per 30 or 40 fish) and the pooled data for the treated and control fish were compared using the Tukey test for multiple comparisons of proportions (Zar 1984). The probability that the observed mortalities were significantly different was taken as $p < 0.05$. Relative percentage survival (RPS) was calculated according to the formula suggested by Amend (1981).

RESULTS AND DISCUSSION

It has been shown by Evelyn & Wong (cited in Evelyn 1988) that coho salmon *Oncorhynchus kisutch* immunized with 10 µg of *Vo* LPS via the IP route, or with 10 µg ml^{-1} of LPS via the IMM method, demonstrated protective immunity towards *Vo* when experimentally challenged. In the present experiment, both the pure CW-LPS and semi-purified EC-LPS were found to elicit protective immunity in juvenile coho salmon, by either the IP or IMM mode of immunization (Table 1). The level of challenge used in this experiment was low and resulted in mortalities similar to those observed in pen-cultured salmonids (Evelyn unpubl. data). Protective immunity in coho vaccinated by IMM declined with decreasing concentrations of EC-LPS, the lowest dose eliciting immunity lying between 1 and 100 ng ml^{-1}. With IP immunization, solid protection was found in coho that received the lowest dose of EC-LPS tested (1 ng). The latter observation suggested that doses of LPS below 1 ng $fish^{-1}$ are immunogenic.

The second experiment was undertaken to investigate the lowest doses of LPS required to induce immunity via the IP and IMM methods under conditions of a strenuous challenge. For IP immunization, the lower limit of LPS required to yield protective immunity was again not determined because even the lowest dose tested (100 pg/4.8 g fish) was sufficient to result in almost complete protection (Table 2). This dose was equivalent to approximately 20 ng LPS kg^{-1} fish. With the IMM method, we found 500 ng ml^{-1} to 1 µg ml^{-1} of LPS resulted in essentially complete protection in fish immersed for either 3 or 30 min. Therefore, at these concentrations of LPS, extending the time of immersion from 3 to 30 min was not useful. However, at the lowest concentration of LPS tested (10 ng ml^{-1}), extending the time of immersion resulted in higher levels of survival. These results suggest that when the vaccine is very dilute, better protection will be achieved by extending the immersion time. In summary, the findings indicate that very small amounts of *Vo* LPS (ca 20 ng kg^{-1} fish) are sufficient to induce virtually complete protection (RPS 85 %) in coho salmon via the IP route. The concen-

Table 1. *Oncorhynchus kisutch*. Immunization of coho salmon with various forms of *Vibrio ordalii* (*Vo*) lipopolysaccharide (LPS). Experiment carried out at 12 to 14 °C with fish averaging 13.7 g; fish were challenged intraperitoneally with 1.1×10^5 viable *Vo* cells 21 d post-vaccination; ensuing mortalities were monitored for 14 d. Abbreviations are: (EC and CW): extracellular and whole cell, respectively; (t1/t2): tanks 1 and 2; (RPS): relative percentage survival. Proportion of mortalities followed by different letters are significantly different at $p < 0.05$ for the specified method of vaccination

Method	LPS		No. dead $tank^{-1}$ (t1/t2)	Total no. dead/ no. challenged	Total dead (%)	RPS (%)
	Form	Dose				
Intraperitoneal injection	Control		2/8	$10/40^a$	25.0	0.0
	EC-LPS	1 ng	0/0	$0/40^b$	0.0	100.0
	EC-LPS	100 ng	0/0	$0/40^b$	0.0	100.0
	EC-LPS	500 ng	0/0	$0/40^b$	0.0	100.0
	CW-LPS	500 ng	0/0	$0/40^b$	0.0	100.0
Immersion	Control		9/8	$17/40^a$	42.5	0.0
	EC-LPS	1 ng ml^{-1}	3/9	$12/40^a$	30.0	29.4
	EC-LPS	100 ng ml^{-1}	1/1	$2/40^b$	5.0	88.3
	EC-LPS	500 ng ml^{-1}	0/0	$0/40^b$	0.0	100.0
	EC-LPS	1 µg ml^{-1}	0/0	$0/40^b$	0.0	100.0
	CW-LPS	1 µg ml^{-1}	0/0	$0/40^b$	0.0	100.0

Table 2. *Oncorhynchus kisutch.* Immunization of coho salmon with *Vibrio ordalii* (*Vo*) extracellular lipopolysaccharide (EC-LPS). Experiment carried out at 12 to 14 °C with fish averaging 4.8 g; fish were challenged intraperitoneally with 1.3×10^5 viable *Vo* cells 21 d post-vaccination; ensuing mortalities were monitored for 14 d. See Table 1 for further details

Method	Dose	No. dead tank^{-1} (t1/t2)	Total no. dead/ no. challenged	Total dead (%)	RPS (%)
Intraperitoneal injection	Control	20/13	33/40[a]	82.5	0.0
	100 pg	2/3	5/40[b]	12.5	84.9
	500 pg	0/0	0/40[c]	0.0	100.0
	1 ng	0/1	1/40[c]	2.5	96.9
	100 ng	0/0	0/40[c]	0.0	100.0
Immersion (3 min)	Control	16/•	16/20[a]	80.0	0.0
	10 ng ml^{-1}	16/12	28/40[a]	70.0	12.5
	100 ng ml^{-1}	8/6	14/40[b]	35.0	56.3
	500 ng ml^{-1}	1/1	2/40[c]	5.0	93.8
	1 µg ml^{-1}	3/1	4/40[c]	10.0	87.5
Immersion (30 min)	Control	11/•	11/20[a]	55.0	0.0
	100 ng ml^{-1}	3/7	10/40[b]	25.0	54.5
	100 ng ml^{-1}	4/5	9/40[b]	22.5	59.1
	500 ng ml^{-1}	0/0	0/40[c]	0.0	100.0
	1 µg ml^{-1}	0/0	0/40[c]	0.0	100.0

• Shortage of tanks precluded the use of 2 replicates for each of the immersion controls

tration of *Vo* LPS resulting in a similar level of protection via the IMM route was 500 ng ml^{-1}, irrespective of the immersion time.

The high immunogenicity shown with *Vo* LPS is consistent with that found for *Va* LPS by Aoki et al. (1984). They showed that *Va* LPS in amounts as low as 10 ng ml^{-1} elicited immunity to *Va* when immersion-administered, a finding that was presaged by the earlier studies of Croy & Amend (1977) who established that crude *Va* preparations could be highly diluted and still result in immunity to *Va*.

No agglutinating antibody titer was detected in any of the samples prior to vaccination or just prior to challenge. The failure to detect agglutinins in our fish following vaccination was not surprising because the amount of LPS administered by injection was small (1 µg fish^{-1} or less) and because vaccination by immersion usually fails to elicit detectable *Va* agglutinins (Aoki et al. 1974, Croy & Amend 1977, Kawano et al. 1984, Sakai et al. 1984). Notwithstanding this, and as will be reported in a separate communication, the plasma from the *Vo* LPS-vaccinated coho salmon (unlike that from the unvaccinated controls) contained *Vo*-specific antibodies because it could be used in passive immunization experiments to protect naive coho salmon from challenge with *Vo* and because this protection could be abrogated by absorbing the plasma with *Vo* cells but not with cells of a serologically unrelated fish pathogen *Renibacterium salmoninarum*. These facts clearly indicate that the anti-*Vo* protection resulting from vaccination with *Vo* LPS was due to a specific immune response.

Exposure of naive juvenile coho to bath challenge with levels of *Vo* cells insufficient to induce vibriosis, resulted in protective immunity (Table 3). Live *Vo* cells in concentrations as low as 2.8×10^1 ml^{-1} resulted in significant protection when only a moderate level of IP challenge was used (42.5 % mortalities in controls). This level of protection was enhanced when the concentration of *Vo* cells in the initial exposure was increased by 100-fold. When the initial concentration was increased to 1×10^6 cells ml^{-1}, coho salmon exhibited solid protection even when the challenge applied was severe enough to kill 90 % of controls.

The above findings suggest that in farmed salmonids, natural challenge with *Vo* cells, at concentrations low enough not to cause disease, will serve to induce immunity against the pathogen and perpetuate the protective effect of an earlier vaccination. Under natural conditions, however, we surmise that a repeated presence of the pathogen on the farm will be required to ensure that a significant proportion of the fish is immunized because it seems probable that only a small proportion of the fish would come in contact with the pathogen during each of its appearances on the farm. Exposure to live *Va* cells has also been found to induce immunity in other salmonids (Braaten & Hodgins 1976, Egidius & Andersen 1979, Evelyn & Ketcheson 1980, Thorburn et al. 1987) but, as Thorburn et al. (1987) have suggested, the value of natural challenge with *Va* may often be reduced because of the farmers' tendency to terminate infections by early interventions using chemotherapy.

Vo-induced immunity obtained in this study was almost certainly elicited by LPS because the LPS prepa-

Table 3. *Oncorhynchus kisutch*. Immunization of coho salmon by bath exposure to live *Vibrio ordalii (Vo)* cells: effect of *Vo* cell concentration on degree of protection. Experiments carried out at 12 to 14 °C with fish averaging 13.5 g; none of the fish exposed to live *Vo* by immersion died or developed signs of vibriosis; they were then challenged intraperitoneally (IP) at indicated times post-exposure with indicated dosages of *Vo* cells; ensuing mortalities were monitored for 14 d. See Table 1 for further details

Vo immersion dose (cells ml^{-1})	No. d prior to challenge	IP challenge (no. *Vo* cells $fish^{-1}$)	No. dead $tank^{-1}$ (t1/t2)	Total no. dead/ no. challenged	Total dead (%)	RPS (%)
Control	21	1.1×10^5	8/9	17/40[a]	42.5	0.0
2.8×10^1	21	1.1×10^5	4/4	8/40[b]	20.0	52.9
2.8×10^3	21	1.1×10^5	0/0	0/40[c]	0.0	100.0
Control	13	1.2×10^5	15/12	27/30[a]	90.0	0.0
1.1×10^6	13	1.2×10^5	0/0	0/40[b]	0.0	100.0

rations tested contained only small amounts of other detectable antigens (1.2 to 2.6% protein). The quantities of LPS required to induce immunity were low, suggesting that the cost of producing anti-*Vo* vaccines should not be prohibitive. The *Vo* findings obtained here parallel those reported for the better studied *Va*, in that immunity to vibriosis could be induced by LPS or by live cells and in that strong immunity could occur in the absence of detectable humoral agglutinins. Whether these observations would hold true for *Vibrio salmonicida*, another important pathogen of salmonids farmed in seawater, remains to be seen.

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