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Nature of the immune response in coho salmon *Oncorhynchus kisutch* following vaccination with *Vibrio ordalii* lipopolysaccharide by two different routes

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ABSTRACT: The question as to whether immersion (=IMM) vaccination stimulates an immune response similar to that yielded by vaccination via the intraperitoneal (=IP) route was examined using *Vibrio ordalii* (Vo) lipopolysaccharide as immunogen and coho salmon *Oncorhynchus kisutch* as test fish. The experimental approach was to determine if the partially purified immunoglobulins in the plasma and mucus from coho salmon vaccinated by each of the above methods were protective to naive coho recipients. Results indicated that the plasma immunoglobulins from IP- and IMM-vaccinated coho protected the naive coho recipients against challenge with live Vo cells. Protection was due to Vo-specific antibodies because the protection could be removed from the transferred material by absorption with Vo cells but not with cells of a serologically unrelated fish pathogen (*Renibacterium salmoninarum*). No protection was transferred with material concentrated from mucus of donors that were IP- or IMM-vaccinated. Overall, our results indicate that both IP and IMM methods of vaccination yield a humoral (systemic) response and that this response is probably the important protective response following vaccination or following natural challenges in the field.

INTRODUCTION

The intraperitoneal (=IP) vaccination of salmonids with *Vibrio anguillarum* (=Va) bacterins elicits a humoral immune response resulting in protection against vibriosis (Harrell et al. 1975, Groberg 1982). This was demonstrated by passively transferring serum from IP-immunized fish to naive fish, the latter then showing protective immunity upon challenge with Va. There was no protection in the recipient fish when the immune serum was absorbed with Va cells prior to the passive transfer, indicating the specificity of the antibody transferred (Harrell et al. 1976). Local immunity also appeared to occur in rainbow trout *Oncorhynchus mykiss* vaccinated with Va bacterins by the IP route because cutaneous mucus from such fish displayed anti-Va activity. However, the activity in the mucus occurred at a much lower level than that in the serum and it was only observed when the Va bacterin contained adjuvant (Harrell et al. 1976).

Bath or immersion (=IMM) vaccination is an effective and convenient way of immunizing fish against several bacterial fish pathogens. However, very little is known about the nature of the immune response(s) elicited (Tatner & Horne 1986), and there is considerable uncertainty as to whether it stimulates the same immune response(s) as that (those) elicited by the IP route. IMM-immunized rainbow trout showed no anti-Va activity in cutaneous mucus (Tatner & Horne 1986) even though studies indicate that lymphocytes capable of producing anti-Va antibodies following IMM vaccination occur in the integument of rainbow trout (St. Louis-Cormier et al. 1984, Pelleteiro & Richards 1988, Magor & Cone 1989). On the other hand, IMM-vaccinated salmonids sometimes show a humoral immune response to Va vaccines (compare, for example, Croy & Amend 1977, Sasaki & Ghoda 1983, Aoki et al. 1984, Kawano et al. 1984, Sakai et al. 1984, Ward et al. 1985, Tatner & Horne 1986, Thuvander et al. 1987, Thorburn & Jansson 1988, Thorburn et al. 1989). When the

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response was positive, it was generally weak. 'High' levels of humoral antibodies, detectable by the enzyme-linked immunosorbent assay, have only been reported once in IMM-vaccinated fish but even in this case the proportion of fish sero-positive by the IMM method was considerably lower than that found for IP-vaccinated fish (Thorburn et al. 1989). Attempts, using the passive transfer approach, to determine whether humoral immunity occurs in salmonids following IMM vaccination have yielded contradictory results. Viele et al. (1980) were successful in transferring immunity to naive rainbow trout with plasma from rainbow trout IMM-vaccinated with Va. On the other hand, Aoki et al. (1984) found the opposite using ayu *Plecoglossus altivelis*.

This study was conducted to determine if vaccination by the IMM route elicits an immune response similar to that induced via the IP route. Would, for example, the serum and mucus from fish vaccinated by each of these routes contain protective antibodies? We examined this question by attempting to transfer immunity to naive coho salmon with plasma and mucus preparations from coho donors immunized by the IP or IMM route with *Vibrio ordalii* (Vo) lipopolysaccharide (= LPS). Vo, like the better known Va, produces a highly immunogenic LPS, which, in our experience (Velji et al. 1990), elicited strong anti-Vo protection in the absence of detectable serum agglutinins.

MATERIALS AND METHODS

Bacterial strains. The Vo isolate (74/48) used for challenging the recipient coho salmon *Oncorhynchus kisutch* was originally isolated from a sockeye salmon *Oncorhynchus nerka* that had died of vibriosis (Evelyn & Ketcheson 1980). The isolate was also used for the preparation of cell-wall LPS (the antigen used for vaccinating fish in the present study) (Velji et al. 1990), and for the absorption of coho plasma and mucus and of rabbit anti-Vo serum (used as reference reagent). Vo cells were grown either on tryptic soy agar (Difco) (=TSA), or in brain heart infusion broth (Difco) (=BHIB), both of which were supplemented with 1 % NaCl. The *Renibacterium salmoninarum* (=Rs) isolate (isolate 384) used for the absorption of the various plasma, mucus, and serum preparations, was isolated from a juvenile chinook salmon *Oncorhynchus tshawytscha* (Evelyn et al. 1986) and was grown on KDM2 medium (Evelyn 1977). Prior to their use for absorbing the plasma, serum, and mucus preparations, Vo and Rs cells were heat-killed (70° C for 30 min) and saline-washed.

Experimental animals. The juvenile coho salmon used in this study were from a local stock (Big Qualicum River) and were reared in well water at the Department of Fisheries and Oceans Rosewall Creek facility. The fish were transported to the laboratory at the Pacific Biological Station at least 10 d prior to use and were held in 800 l tanks, supplied with flowing dechlorinated city water. The fish were gradually acclimatized to a temperature of 13 ± 1 °C. Donor salmon were from the 1988 hatch; recipient coho were from the 1989 hatch.

Active immunization of donor coho. Coho salmon (average weight 80 g) used as source of plasma and mucus were divided into 3 groups of 17 fish each. The first group served as unvaccinated controls; the second was IMM-immunized for 15 min in 2 l of aerated fish-culture water containing 5 µg of Vo LPS ml^{-1}; the third was IP-immunized with 1 µg of Vo LPS $fish^{-1}$. Vo LPS was quantitated by the chromogenic limulus amoebocyte lysate method (Whitaker M. A., Bio-Products, Walkerville, MD). Each group was maintained separately for 21 d post vaccination in tanks supplied with flowing dechlorinated city water at 13 ± 1 °C. After this time, the fish were anesthetized with 2-phenoxy-ethanol (1:7000) and the cutaneous mucus was collected by a combination of flushing (with 0.15 M, pH 7.4 phosphate buffered saline = PBS) and suction. This method proved to be easier for harvesting mucus than the other methods described to date (Harrell et al. 1976, Cipriano 1986, Lobb 1987). Immediately after mucus collection, blood samples were obtained from the severed caudal peduncle in heparinized Caraway tubes. The blood was centrifuged and the plasma harvested. Mucus and plasma samples were pooled separately, according to treatment group.

Rabbit anti-Vo serum production. Antiserum against Vo was produced by intravenous injection of a New Zealand white female rabbit with boiled Vo cells (presumably containing the heat stable LPS antigen) using the immunization schedule of Toranzo et al. (1987) with slight modifications. Vo cells were grown in BHIB, supplemented with 1 % NaCl, for 48 h at 21 °C and then pelleted by centrifugation. Cells were washed twice with PBS and then suspended in PBS to an optical density of 6.0 at 540 nm. They were then boiled for 1 h. The initial immunization schedule was similar to that of Toranzo et al. (1987). The rabbit was allowed to rest for 2 wk after which it was boosted with the antigen. The blood was collected 1 wk later from the marginal ear vein and was allowed to clot. The resulting anti-Vo serum was used as described in the ensuing sections.

Preparation of materials to be passively transferred. Coho mucus:PBS washings were spun in a clinical centrifuge at 3000 × *g* for 10 min to pellet any particulate material. Some 250 ml of dilute mucus from each treatment group were then concentrated by positive pressure in an ultrafiltration cell equipped with a

YM100 filter (Amicon Corp., Danvers, MA). Coho mucus and plasma were then each treated with 50 % ammonium sulphate (w/v, final concentration) to precipitate any immunoglobulins present. The precipitate was redissolved in PBS and dialyzed against PBS. Dilute mucus preparations were then concentrated to the stage where they contained ca 0.8 mg protein per ml (based on absorbance at 280 nm; see Scopes 1982). Plasma preparations were adjusted with PBS to contain approximately the same concentration of protein (1.0 mg ml^{-1}). Mucus and plasma preparations were then filter-sterilized using a 0.22 µm pore size Millipore membrane and stored at −20 °C until used. Rabbit antiserum was treated with ammonium sulphate to a final concentration of 45 % (w/v), and the resulting precipitate was dissolved in PBS and then dialyzed against PBS. This was followed by dialysis against 0.075 M PBS (half strength PBS) and loading on a DEAE Sephacel ion exchange column that had previously been equilibrated with the same buffer. The protein fraction (monitored at 280 nm) that ran through the column in the void volume represented the immunoglobulin and was collected (Clark & Adams 1977). Based on absorbance at 280 nm, the protein content of the fraction was 0.54 mg ml^{-1} (Scopes 1982). The immuno-globulin preparation was diluted 1/10 with PBS prior to absorption.

Absorption of coho mucus, coho plasma, and rabbit serum. The immunoglobulin-enriched protein fractions obtained from vaccinated and unvaccinated coho and from the vaccinated rabbit were each divided into 3 equal aliquots. One aliquot of each was left untreated, i.e. unabsorbed. The second and third aliquots were absorbed 3 times with Vo and Rs cells, respectively. Prior to their use, Vo and Rs cells were suspended separately in PBS to a calculated optical density of 11.0 at 540 nm. The cells were then pelleted by centrifugation and the supernatant was replaced with an equal volume of the preparation to be absorbed. The pelleted cells were resuspended by vortexing and the mixtures were incubated at 15 °C for 7 h with occasional mixing. After absorption, cells were removed by centrifugation and supernatants were absorbed 2 more times. Absorbed preparations were stored at 4 °C until used.

Passive transfer. Juvenile coho (average weight 5.2 g) were anesthetized as already described and then injected IP with 0.1 ml of one or the other of the above absorbed or unabsorbed preparations. Eighteen coho were used for each preparation, and fish receiving the various preparations were held in separate 35 l tanks supplied with dechlorinated city water at 13 ± 1 °C. Each fish was challenged IP 5 h later with 0.1 ml of PBS containing 7.7×10^5 live Vo cells. Resulting deaths in the challenged fish were monitored for 14 d, by which time deaths appeared to have ceased. Kidney samples from 10 % of the dead fish were plated on TSA supplemented with 1 % NaCl to verify the cause of death. Identification of Vo growth on the plates was accomplished using Vo-specific rabbit antiserum (Microtek, Sidney, BC) in a slide agglutination test.

Statistical analysis. Mortalities among the various treatment groups were compared using the log-likelihood ratio or G-test to determine whether they were significantly different (Sokal & Rohlf 1981). We were particularly interested in determining whether plasma or mucus preparations from vaccinated fish yielded survival superior to that from unvaccinated fish or to that of fish receiving saline. We were also interested in determining whether plasma or mucus preparations from IMM- and IP-vaccinated fish yielded different survivals and whether absorption of the preparations with Vo or Rs cells affected the survivals.

RESULTS AND DISCUSSION

When plasma was the preparation passively transferred, mortality levels in the Vo-challenged recipient fish depended on whether the fish donating the plasma had been immunized and on whether homologous antigen had been used to absorb the transferred plasma preparation ($p < 0.01$) (Table 1). Fish receiving material from IP- or IMM-vaccinated donors survived significantly better ($p < 0.01$) than those receiving material from unvaccinated donors or than those receiving only saline. This clearly indicates that vaccination by both methods results in the production of humoral factors (almost certainly immunoglobulins, as discussed later) capable of conferring protection against Vo. There did not appear to be any differences in mortality when IP and IMM methods of vaccination were compared ($p > 0.05$). In active immunization, one would have expected vaccination by the IP route to yield stronger protection than that obtained via the IMM route (Evelyn 1984). However, with the passive immunization approach used, we were not measuring the relative strengths of active immunity produced by the 2 methods. We measured only the fact that, at 21 d post vaccination, the levels of the protective factors in the plasma of fish vaccinated by each method were adequate to protect against the challenge dose used.

The results listed in Table 1 also show that the protective effect of plasma preparations from immunized donor coho could be removed by absorbing the preparation with the homologous antigen (Vo cells) ($p < 0.01$) but not with a serologically unrelated antigen (Rs cells) ($p > 0.05$). The protection conferred by the plasma preparations was therefore Vo-specific. Protection obtained with serum preparations derived from the Vo-vaccinated rabbit also showed a similar specificity:

Table 1. *Oncorhynchus kisutch*. Passive immunization with plasma preparations from donor coho immunized with *V. ordalii* (Vo) lipopolysaccharide (LPS): effect of vaccination method and of absorbing the plasma preparations with Vo and *R. salmoninarum* (Rs) cells[a]

Preparation transferred	Immunization method of donor[b]	No. fish dead/ no. challenged[c]
Coho plasma (non-absorbed)	None (control)	18/18
	IMM	1/18
	IP	3/18
Coho plasma (Vo-absorbed)	None (control)	18/18
	IMM	18/18
	IP	18/18
Coho plasma (Rs-absorbed)	None (control)	16/18
	IMM	5/18
	IP	1/18
Rabbit serum[d] (non-absorbed)	IV	0/18
Rabbit serum (Vo-absorbed)	IV	18/18
Rabbit serum (Rs-absorbed)	IV	0/18
Phosphate buffered saline (PBS)	None	15/18

[a] Recipient fish averaged 5.2 g and were held at 13 ± 1 °C; they were challenged intraperitoneally with 7.7×10^5 live Vo cells 5 h after receiving the test preparation; ensuing deaths were monitored for 14 d

[b] Donor coho averaged 80 g and were vaccinated by a 15 min immersion in water containing 5 µg of Vo LPS ml^{-1} (= IMM method), by intraperitoneal injection of 1 µg Vo LPS $fish^{-1}$ (IP method), or by intraperitoneal injection of PBS (controls)

[c] Groups with 0 to 5 fish dead are significantly different from groups with 15 to 18 fish dead ($p < 0.01$). Groups with 0 to 5 fish dead are not significantly different from each other ($p > 0.05$). Groups with 15 to 18 fish dead are not significantly different from each other ($p > 0.05$)

[d] Rabbit anti-Vo serum preparations were used for comparative purposes; for details of their preparation consult text

protection could be abrogated by absorption with Vo cells ($p < 0.01$) but not with cells of a heterologous pathogen (Rs) ($p > 0.05$). Taking into account the specificity of the protective factor, the fact that it was induced by vaccination, and the fact that it was precipitable with 45 to 50 % ammonium sulphate, it seems likely that the factor was immunoglobulin.

The foregoing results for the IP-vaccinated coho are consistent with the results obtained for Va by Harrell et al. (1975) and Groberg (1982) who used serum from IP-vaccinated rainbow trout and coho, respectively, to protect naive recipient fish against challenge with Va – a protection that was shown to be specific (Harrell et al. 1975). Our results with IMM-vaccinated coho also agree with the findings of Viele et al. (1980) for rainbow trout that had been IMM-vaccinated with Va bacterins: serum from such fish protected naive rainbow trout recipients against Va. In contrast, Aoki et al. (1984) reported that serum from ayu IMM-vaccinated with Va LPS was not protective for naive ayu recipients.

No Vo agglutinin titers were detected in the plasma samples from any of the coho, vaccinated or otherwise, in the present experiment (data not shown). The lack of detectable agglutinins in the vaccinated fish was probably due to the inherent insensitivity of the agglutination technique and the small amounts of antigen administered in our experiments. Other workers, using higher levels of antigen, have certainly observed humoral agglutinins in fish vaccinated by the IP route with Va bacterins (Harrell et al. 1976, Aoki et al. 1984) or with Va LPS (Salati et al. 1989). The agglutinin titers were highest when adjuvant was present in the vaccine (Harrell et al. 1976). The lack of detectable agglutinins observed in our experiments is more in line with what has been reported in the literature for fish vaccinated against Va by the IMM method: such fish contained low to no detectable humoral agglutinins (Aoki et al. 1984, Tatner & Horne 1986) or anti-Va antibodies (Thorburn & Jansson 1988); further, when IMM-induced humoral antibodies were detected, they occurred at lower levels and more sporadically than in IP-vaccinated fish (Thorburn et al. 1989) and they occurred in highest titers only when the time of immersion in the vaccine was prolonged (Sasaki & Ghoda 1983). It seems, therefore, that both IP and IMM methods of

vaccination elicit the production of humoral antibodies in fish but that the titers of these antibodies are related to the amount of antigen that enters the fish to stimulate the response. With IMM vaccination, the amount of antigen entering the fish is normally less than that by the IP route and the degree of stimulation of the immune system in IMM-vaccinated fish would be correspondingly less intense (Zapata et al. 1987).

Mucus preparations from coho vaccinated by the IP and IMM routes did not prove protective ($p > 0.05$) for naive coho recipients (Table 2) and contained no detectable anti-Vo agglutinins (data not shown). This lack of any evidence of protective immunoglobulins in the mucus was not particularly surprising. In their studies with IP-vaccinated rainbow trout, Harrell et al. (1976) were only able to demonstrate the presence of mucous antibodies when adjuvant-containing Va vaccines were used and only in fish with unusually high humoral anti-Va titers (> 131, 072). Further, although Tatner & Horne (1986) were able to detect anti-Va and anti-Vo agglutinins in the serum of rainbow trout IMM-vaccinated against Va and Vo, they were unable to do so with mucus.

Our negative results with coho mucus may have been a function of the amount of Vo antigen used in our studies. Or, perhaps the immunoglobulin-secreting cells that have been reported as occurring in the salmonid integument (St. Louis-Cormier et al. 1984, Pelleteiro & Richards 1988, Magor & Cone 1989) require repeated dosing to induce them to secrete protective titers of antibody. If so, the cutaneous antibody secreting system in coho must be far less easily stimulated than that in channel catfish (Ourth 1980, Lobb 1987). Whatever the explanation, we conclude that mucus-mediated immunity to Vo in coho is not likely to be nearly as important as systemically (humorally) mediated immunity, particularly under challenge conditions experienced in nature or in netpens. The basis of this conclusion is that doses of Vo LPS as high as 1 µg, administered by IP injection, failed to produce factors in coho mucus protective against Vo. This amount of LPS is equivalent to ca 5×10^6 Vo cells (Velji unpubl.) – a dose of Vo cells that in our laboratory has regularly resulted in 100 % mortality when injected into juvenile coho salmon and that is far in excess of the number of living Vo cells needed to induce measurable anti-Vo immunity in coho by bath exposure (Velji et al. 1990). Whether the relative unimportance of the cutaneous immune system in coho for Vo holds true for other salmonids and fish pathogens remains to be determined.

Table 2. *Oncorhynchus kisutch*. Passive immunization with mucus preparations from donor coho immunized with *V. ordalii* (Vo) lipopolysaccharide (LPS): effect of vaccination method and of absorbing the mucus preparations with Vo and *R. salmoninarum* (Rs) cells[a]

Preparation transferred	Immunization method of donor[b]	No. fish dead/ no. challenged[c]
Coho mucus (non-absorbed)	None (control)	15/18
	IMM	18/18
	IP	17/18
Coho mucus (Vo-absorbed)	None (control)	18/18
	IMM	16/18
	IP	18/18
Coho mucus (Rs-absorbed)	None (control)	18/18
	IMM	14/18
	IP	14/18
Phosphate buffered saline (PBS)	–	15/18

[a, b] See details under Table 1
[c] None of the groups showed deaths that were significantly different from each other

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