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Trials with an orally and immersion-administered β-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha*

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ABSTRACT: A study was conducted to examine the effectiveness of a commercially available β-1,3 glucan, VitaStim-Taito (VST), to stimulate immunity in juvenile chinook salmon *Oncorhynchus tshawytscha* against the bacterium *Aeromonas salmonicida* (As) causing furunculosis in salmon. VST had earlier been shown to be capable of elevating the potency of an anti-furunculosis vaccine when administered by injection. In the present study, 2 routes of administration were evaluated for their effectiveness: oral delivery and immersion. In the oral administration trials, VST in its crudest form was mixed (without the As vaccine) into a steam-pelleted diet at a rate of 0, 0.01, 0.1, and 1.0 %. The 4 diets were fed to test fish at 2 % of body weight d^{-1}. After 7 d on the diets, the fish were bath-challenged with As, the fish being maintained on the test diets for the rest of the experiment. VST added to the diet at either 0.1 or 1.0 ‰ resulted in significant protection against the As challenge. In the immersion trials, an As vaccine was administered to the test fish either alone or in combination with a pure or a crude form of VST. The vaccine and the VST were also administered alone. Following a bath challenge with virulent As at 21 d post-vaccination, no significant protection was noted in any of the groups tested, indicating that VST was inactive as an immunopotentiator by this route. The fish were, however, immunocompetent because protection occurred when they were injected with the vaccine alone.

KEY WORDS: *Aeromonas salmonicida* · Furunculosis · Immunomodulation · Vaccination

INTRODUCTION

Research on vaccines against furunculosis, caused by *Aeromonas salmonicida* (As), has had a long history (see Duff 1942) compared to that on vaccines against other fish diseases. Despite this, available furunculosis vaccines do not always yield satisfactory results (Ellis 1988) and fish culturists still often find it necessary to use antibiotics to control the disease. Chemotherapy, however, has several drawbacks, among them the risk of generating antibiotic resistant pathogens and the problem of drug residues in the flesh of treated fish destined for human consumption. Alternatives to chemotherapy are therefore highly desirable.

It has long been recognized that immunomodulators may be used to more fully exploit the defence systems of mammals (Fenichel & Chirigos 1984) but researchers have been slow to investigate the immunopotentiating possibilities of these agents in fish. Recently, however, Olivier et al. (1985a) and Kitao & Yoshida (1986) showed that Freund's complete adjuvant and a synthetic peptide (FK-565), respectively, elicited protection in juvenile salmonids against As. A number of other recent studies have also established that enhanced immune responses can be generated in fishes using a variety of other immunomodulators, including a tunicate extract (Davis & Hayasaka 1984), levamisole (Siwicki 1987), and the β-1,3 glucans (Yano et al. 1989, 1991, Robertsen et al. 1990). The possibilities for use of immunomodulators in fish are therefore promising.

The factors in fish responsible for immunity to furunculosis are not fully understood. There are some indi-

cations, based on passive immunization experiments with antibodies produced in salmonids, that antibodies may play a role in the process (Spence et al. 1965, Olivier et al. 1985b, Ellis et al. 1991). However, the general view has been that there is a poor association between the production of As antibodies and resistance to As (Michel 1979, Cipriano 1982, 1983, Olivier et al. 1985a) and that resistance to As is better correlated with the cellular immune response (Smith et al. 1980). Indeed, Olivier et al. (1985a) showed that the increased resistance to As elicited by Freund's complete adjuvant was the result of increased bacterial killing by macrophages. These data suggested that immunomodulators capable of activating macrophages might be of prophylactic value against As.

In a recent study, Nikl et al. (1991) showed that 3 of 7 immunomodulators tested consistently resulted in enhanced immunity to As in salmonids when injected. One of these substances was the commercially available VST. In the study reported on here, we investigated whether VST showed promise against As when administered by the more convenient (and less stressful) methods of feeding and immersion.

MATERIALS AND METHODS

Experimental animals. The chinook salmon used in these experiments averaged 3.7 and 6.5 g (see below). The fish were reared on a commercial ration in 9 °C pathogen-free well water at the nearby Department of Fisheries and Oceans' Rosewall Creek facility. At least 2 wk prior to the start of each experiment, the fish were transferred to the laboratory at the Pacific Biological Station where they were acclimated to their new conditions and to ambient water temperatures of 13 to 15 °C (1 °C d^{-1}). The water supply in the laboratory was dechlorinated municipal water; it had the characteristics described by Bell et al. (1984) and was ultraviolet light-treated in the laboratory prior to use with the fish.

The pathogen. The isolate of As used in the experiments (# 76-30; Olivier et al. 1985b) was grown in trypticase soy broth (TSB) (Difco, Detroit, MI, USA) for 4 d at 15 °C. The resulting culture was used either to bath-challenge (see later) the variously treated fish or was used as a vaccine after inactivation with 0.3 % (v/v) formalin. The isolate was A-layer positive by the Coomasie brilliant blue test (Cipriano & Bertolini 1988) and autoaggregated readily; its virulence for salmon was maintained by frequent passage through coho salmon *Oncorhynchus kisutch*.

Challenge protocol and cause of death. The challenges were accomplished by the bath method. in the procedure, fish were immersed for 15 min in the aerated, water-diluted, As TSB broth culture, a viable count of the challenge suspension being made on tryptic soy agar (TSA) (Difco) by the drop-inoculation method (Miles & Misra 1938). Of the fish dying following challenge, 10 % were selected at random so that the cause of death could be verified. Furunculosis was considered to be the cause of death when smears of kidney material showed the presence of short, Gram-negative rod-shaped bacteria with slightly polar staining characteristics and when pure cultures of the brown pigment-producing bacterium were recovered on TSA.

VST-containing diets. Special diets containing 4 different levels of VST (0, 0.01, 0.1, and 1.0 %) were prepared so that the efficacy of VST administered via the oral route could be evaluated. The crudest form of VST available (Taito Co. Ltd., Tokyo, Japan) (VST MW = 1 to 2 $\times$ 10^6) was used because this form of VST has the lowest production cost and the highest molecular weight. Higher molecular weight forms of VST have greater biological (e.g. anti-tumor) activity (Kojima et al. 1986). The preparation used consisted of the dried, spent broth culture (containing excreted VST) and the colloidally pulverized VST-containing mycelia of the source fungus *Schizophyllum commune*. A basal diet was prepared by first mixing the dry ingredients (Table 1) together for 15 min to ensure homogeneity. Next, the liquid constituent (herring oil) was added, and mixing was continued for an additional 15 min. The vitamin and mineral supplements (Table 1, footnotes a and b, respectively) were prepared separately

Table 1. Composition of the basal diet used in the oral administration trials with VitaStim-Taito

Constituent	g kg^{-1} diet
Steam dried herring meal	558.7
Dried whey	84.6
Blood flour	48.2
Euphasids (whole, frozen)	21.0
Wheat middlings	126.2
Herring oil	14.3
Vitamin supplement[a]	18.4
Mineral supplement[b]	18.4
Choline chloride (60 % active ingredient)	4.6
Ascorbic acid	1.8
Permapell (TM) (pellet binder)	13.8

[a] Per kg diet: D-calcium pantothenate 0.178 g, pyridoxine hydrochloride 0.0414 g, riboflavin 0.0582 g, niacin (added last) 0.280 g, folic acid 0.0212 g, thiamine mononitrate 0.0374 g, biotin 0.138 g, hetrazone 0.1068 g, vitamin B_{12} 0.055 g, Vitamin E 1.105 g, vitamin D3 0.00442 g, vitamin A 0.0184 g, inositol 0.368 g, α-cellulose 16.001 g

[b] Per kg diet: $MnSO_4 \cdot H_2O$ 0.212 g, $ZnSO_4 \cdot 7H_2O$ 0.202 g, $CoCl_2 \cdot 6H_2O$ 0.0037 g, $FeSO_4 \cdot 7H_2O$ 0.344 g, KIO_3 0.0155 g, NaF 0.0204 g, Na_2SeO_3 0.00020 g, NaCl 3.510 g, $MgSO_4 \cdot 7H_2O$ 2.948 g, α-cellulose 11.139 g

with α-cellulose as the carrier. Additional premixes were prepared containing the desired levels of VST, with α-cellulose forming the non-VST volume of the premix. Both VST and α-cellulose are indigestible in salmonids, thus avoiding the possibility that the nutritional status of the 4 test diets varied. The premixes were added to the basal diet and mixing was again continued for 15 min. The diets thus prepared were identical to one another except for the amount of VST present. Each of the diets was then steam-pelleted (maximum temperature of 95 °C) and allowed to cool and dry. Herring oil was then sprayed onto the surface of the pellets (77.1 g kg^{-1}) with an airless spray gun while rotating the pellets in a motorized cement mixer. The diets were then stored in tightly sealed plastic bags at −20 °C until needed.

Experimental protocol: oral administration of VST. Groups of 50 chinook salmon averaging 3.7 g were placed in 30 l tanks supplied with flowing fresh water at 15 °C for 3 wk. During this period the fish were fed the VST-free (basal) diet to allow them to adjust to it. At this point, the fish were switched to the test diets such that each diet was fed to a different group of fish (3 replicate groups per diet). The various test groups were maintained on their respective diets until the end of the experiment, the diets being fed at ca 2 % of fish body weight d^{-1}. To prevent errors due to the presence of anorexic fish, only the 40 most robust fish in each group were challenged. The fish were bath-challenged in 6.8×10^4 live As cells ml^{-1} after they had been fed the test diets for 7 d.

Experimental protocol: Immersion administration of VST. Fifty chinook salmon averaging 6.5 g were immersed in well-aerated baths of various experimental vaccines for 15 min (see below). A 15 min exposure period was selected because it had been reported as adequate for antigen uptake (Tatner 1987). Two forms of VST were tested, the highly purified and partially depolymerized form of VST (VSTp) (MW 4.75×10^5) and the crude form of VST (VSTm) (MW 1 to 2×10^6), mentioned earlier. Fish were exposed to either water (the controls), VSTp (100 mg l^{-1}), VSTm (150 mg l^{-1}), As vaccine, As vaccine plus VSTp (100 mg l^{-1}), or As vaccine plus VSTm (150 mg l^{-1}). The As vaccine consisted of a 10-fold dilution (in water) of the 4-d-old, formalin-killed As TSB culture, described earlier. No anesthetic was used prior to or during the immersion treatment because anesthesia had been shown by various workers to adversely effect the outcome of vaccination by immersion (Evelyn 1984). As a check on the immunocompetence of these fish, an additional group of 50 fish was injected intraperitoneally with 0.1 ml of the undiluted As vaccine. The fish in this study received a commercially available salmon feed which was withheld from the fish for 2 d before and 2 d after vaccination.

Following immersion vaccination, the variously treated groups were placed in 40 l freshwater tanks (13 °C) for 2 d to allow them to shed any surface-borne VST or As antigens and to provide us with the opportunity to fin-clip them to denote the treatment received. Fish were anesthetized with 2-phenoxyethanol (1:7000) for the procedure. Following this, fish in each treatment were distributed equally between two 135 l tanks where they were held for 21 d at 13 °C to permit time for an immune response to develop. The fish were then bath-challenged with 2.5×10^5 live As cells ml^{-1}.

Statistical analyses. The mortality data from each of the various replicate groups were first subjected to chi-squared heterogeneity testing to determine if it was legitimate to pool results. In cases where the chi-square test was inappropriate, the Fisher's exact test was employed to determine significance. The statistical tests are described in Zar (1984).

RESULTS AND DISCUSSION

Results from the VST feeding experiment, summarized in Table 2, showed that it was legitimate to pool

Table 2. *Oncorhynchus tshawytscha*. Survival of juvenile chinook salmon fed diets containing various levels of crude VitaStim-Taito (VSTm) and bath-challenged with *Aeromonas salmonicida* after 7 d on the diets. Trial was conducted in fresh water at 15 °C; fish were fed the various diets at 2 % of the fish body weight d^{-1} for the duration of the trial; all dead fish sampled died of furunculosis; challenged fish were observed for 21 d and the trial was terminated when deaths had ceased for 7 d. Repl.: replicate

VSTm conc. in diet	Repl. no.[a]	No. dead/ no. treated	Pooled replicates	% Mortality[b]
0	1	10/40	20/120	16.7[A]
	2	5/40		
	3	5/40		
0.01	1	15/40	33/120	27.5[A]
	2	10/40		
	3	8/40		
0.1	1	2/40	2/120	1.7[B]
	2	0/40		
	3	0/40		
1.0	1	2/40	7/120	5.8[B]
	2	4/40		
	3	1/40		

[a] Heterogeneity tests showed that replicate data could be pooled and then analyzed

[b] Values with different superscript letters are significantly different from one another ($p < 0.05$; Chi-square test or Fisher's exact test, as appropriate)

the data obtained for replicates. More important, however, a comparison of the pooled replicate data indicated that significant protection ($p < 0.05$) occurred when VST was fed, although only at the 2 highest dosages (0.1 and 1.0 %) tested. VST thus shows promise for the prophylaxis of furunculosis via the oral route, a route that hitherto has only been evaluated with the *Sacchanomyces cerevisiae* glucan and that yielded promising but inconsistent results (Raa et al. 1992).

Our results thus confirm that the feeding of glucans has potential as a disease control procedure, at least with respect to furunculosis. It should be pointed out, however, that the severity of our As challenge, while representative of challenges sometimes experienced on salmon farms, was not as high as has been recommended for potency testing of vaccines in the laboratory (see Amend 1981). Further testing with fed VST will therefore have to be done to establish the strength of the protection achievable by this route and to delineate the feeding regimes yielding the best results.

A desirable feature of VST is that it is heat-stable enough to survive the steam-pelleting process. Apparently, VST retains its triple helix structure, and thus its biological activity, at temperatures up to 135 °C (Yanaki et al. 1983, 1985).

In the feeding tests, no As vaccine was included in the diet because it was felt that much of the value of the vaccine would be destroyed in the gastrointestinal tract by the digestive enzymes (proteases) present. Protective antigens produced by As are heat sensitive (McCarthy et al. 1983) and thus have long been considered to be proteins, a fact that has now been clearly established by Ellis et al. (1991). We are now aware, however, that a promising orally administered anti-As vaccine is being field-tested (Paterson et al. 1992), indicating, perhaps, that a practical method has been found to shield the protective antigens from the action of the digestive tract enzymes. If such a vaccine functions in combination with VST to produce an enhanced immune response to As akin to that observed when both are administered by injection (Nikl et al. 1991), this could provide the basis of a convenient and effective anti-furunculosis vaccine. The new As vaccine would, however, have to be incorporated into the feed by some means other than steam-pelleting because, as mentioned above, the protective As antigens are heat labile.

Finally, based on work done by others with VST and other glucans, it appears likely that the immunity produced in our feeding study would have been non-specific. The glucans appear to mediate their effect by activating a number of non-specific defence mechanisms, including the increased production of lysozyme (Engstad et al. 1992, Jørgensen et al. 1993) and the activation of macrophages (Raa et al. 1992, Jørgensen et al. 1993) and the alternative complement pathway (Yano et al. 1991) – mechanisms that would result in broad spectrum immunity. Certainly, this was the finding of Robertsen et al. (1990) and Raa et al. (1992) who together reported enhanced resistance to 4 different bacterial fish pathogens in Atlantic salmon *Salmo salar* following administration of the *S. cerevisiae* glucan and of Yano et al. (1991) who reported resistance to 2 different bacterial fish pathogens in carp following injection with various glucans, including VST.

Results obtained with immersion-administered VST were not as encouraging (Table 3) because neither VSTp nor VSTm yielded any protection. This held true whether or not the As vaccine was also present. The As vaccine was also without effect when administered alone by immersion. Clearly, however, the fish involved in the test were immunocompetent because they developed a significant protective immune response when the As vaccine was administered by injection. Taken overall, the results suggest that VST

Table 3. *Oncorhynchus tshawytscha*. Survival of juvenile chinook salmon immersion-vaccinated with VitaStim-Taito in pure (VSTp) and crude (VSTm) form with and without an *Aeromonas salmonicida* (As) vaccine (V) and bath-challenged 21 d later with As. Trial was conducted in fresh water at 13 °C; all dead fish sampled died of furunculosis; challenged fish were observed for 18 d and the trial was terminated when fish had ceased to die for 7 d

Replicate no.[a]	Treatment	No. dead/ no. treated	% Dead	RP[b]
1	Water	16/23	70^{A}	1.0
	VSTp only	16/21	76^{A}	0.9
	VSTm only	18/24	75^{A}	0.9
	V only	14/21	67^{A}	1.0
	VSTp + V	14/24	58^{A}	1.2
	VSTm + V	17/24	71^{A}	1.0
	V (injected)[c]	5/23	22^{B}	3.2
2	Water	9/23	39^{A}	1.0
	VSTp only	10/22	45^{A}	0.9
	VSTm only	7/25	28^{A}	1.4
	V only	11/24	46^{A}	0.9
	VSTp + V	10/25	40^{A}	1.0
	VSTm + V	6/24	25^{A}	1.6
	V (injected)[c]	3/24	13^{B}	3.0

[a] Replicates 1 & 2 were affected differently by the challenge and are thus analyzed separately; values with different superscript letters are significantly different from each other ($p < 0.05$; Chi-squared test or Fisher's exact test, as appropriate)

[b] RP = relative potency: % mortality (control)/ % mortality (test)

[c] As vaccine was injected to ensure contact with the immune system so that immunocompetence of the test fish could be assessed

and As antigens are not readily taken up by fish via the immersion route and that the oral route may be the approach to investigate further

In presenting the results of the challenge following immersion vaccination in Table 3, we found that identical challenges in the replicate tanks yielded poorer survival in one of the replicates than in the other. So that the results in the tanks could be compared directly, we also expressed the results in terms of relative potency (Amend 1981).

In the immersion vaccination experiment, 6.5 % of the fish died within a few days of vaccination and fin-clipping. Deaths occurred in most of the treatment groups but no bacteria were seen in kidney smears of these fish and As was not isolated from their kidneys using TSA. Because deaths occurred even in the groups exposed to water alone, it was concluded that these deaths were due to handling stress brought on by the vaccination and fin-clipping procedures. As recommended by Finney (1971) and Amend (1981), these deaths were not considered when calculating the proportions of fish dying and surviving in the experiment.

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