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Immunogenicity of synthetic peptides representing antigenic determinants on the infectious hematopoietic necrosis virus glycoprotein

E. Emmenegger[1,3,*], M. Landolt[1], S. LaPatra[2], J. Winton[3]

[1]School of Fisheries, PO Box 357980 University of Washington, Seattle, Washington 98195, USA
[2]Clear Springs Foods, Inc., PO Box 712, Buhl, Idaho 83316, USA
[3]Northwest Biological Science Center, 6505 N.E. 65th St., Seattle, Washington 98115, USA

ABSTRACT: Three peptides, P76, P226, and P268 representing 3 putative antigenic determinants on the glycoprotein of infectious hematopoietic necrosis virus (IHNV), were synthesized and injected into rainbow trout *Oncorhynchus mykiss* to assess their immunogenicity. Antisera extracted from the immunized trout were analyzed using an enzyme linked immunosorbent assay (ELISA) for the presence of antibodies that could bind to the peptides or to intact virions of IHNV. The antisera were also tested for neutralizing activity against IHNV by a complement-mediated neutralization assay. In general, recognition of the peptides and IHNV was low and only a few antibody binding patterns were demonstrated. Antisera from fish injected with P76 constructs recognized the homologous peptide more than the heterologous peptides, whereas antisera from fish inoculated with either P226 or P268 constructs recognized P76 equally, or better, than the homologous peptide; however, there was a high degree of individual variation within each treatment group. Neutralization activity was demonstrated by serum from a single fish injected with one of the peptides (P268) and from 7 of 10 positive control fish infected with an attenuated strain of IHNV. Possible explanations for the dichotomous immune responses are discussed. These results indicate we need to improve our overall understanding of the fish immune system in order to facilitate the development of an efficacious vaccine against IHNV

KEY WORDS: IHNV · Immunity Rainbow trout · Synthetic peptides · Vaccine

INTRODUCTION

Infectious hematopoietic necrosis (IHN) is a viral disease affecting salmonid fishes. Mortalities are especially high among cultured fry, and in some cases losses exceed 90% (Leong et al. 1988). No effective control measures against the virus currently exist, other than avoidance and destruction of infected stocks (Winton 1991). Survivors of IHN are resistant to re-infection by the causative agent, infectious hematopoietic necrosis virus (IHNV), indicating that immunity against the virus might be induced by an effective vaccine (Amend 1976). However, attempts to control IHN by inducing immunity with vaccines have not been completely successful. An attenuated strain of IHNV was able to protect vaccinated sockeye *Oncorhynchus nerka* and chinook *O. tshawytscha* for up to 110 d (Fryer et al. 1976), but caused unacceptable levels of mortalities in some stocks of vaccinated rainbow trout *O. mykiss* (Rohovec et al. 1981). Protein sub-unit vaccines against IHNV were reported to provide limited protection (Gilmore et al. 1988, Xu et al. 1991, Noonan et al. 1995), but have not been shown to provide long-term protection, which is a requirement for an IHNV vaccine to be effective (Leong & Fryer 1993, Winton 1996).

IHNV is a rhabdovirus comprised of 5 structural proteins (Morzunov et al. 1995). The glycoprotein is the primary antigen responsible for eliciting a protective immune response (Engelking & Leong 1989). Early efforts to map regions on the glycoprotein that are involved with immunity suggested that 3 antigenic

*E-mail: evi_emmenegger@nbs.gov

domains existed at amino acids 270–340, 336–444, and 454–469 (Gilmore et al. 1988, Mourich & Leong 1991, Xu et al. 1991). Huang (1993) used a panel of neutralizing monoclonal antibodies (MAb) to isolate a series of neutralization-resistant mutants. Sequencing of the entire glycoprotein (G) gene of 6 of these mutants revealed that they differed from the wild-type by a single amino acid substitution. The substitution sites were located at amino acids (aa) 81, 230–231, and 272–276. The results of the study indicated the presence of 3 possible antigenic domains containing neutralizing epitopes. To investigate the immunogenicity of these antigenic sites identified by Huang (1993), we produced synthetic peptides representing each of the 3 probable antigenic domains and used these to immunize rainbow trout.

Synthetic peptides can be used to characterize a suspected antigenic site or to serve as a sub-unit vaccine (Brown et al. 1993). The existence of a B-cell epitope on the envelope protein (gp46) of human T-lymphotrophic virus type 1 (HTLV-1) was confirmed by analyzing sera from rabbits injected with a peptide construct (Env-5) mimicking the epitope (Lairmore et al. 1992). The Env-5 anti-sera recognized the Env-5 peptide and the native gp46 protein in an enzyme linked immunosorbent assay (ELISA) and in an immunofluorescence assay. Peptide homologs of a linear epitope (G5) on the rabies virus glycoprotein induced production of virus neutralizing antibodies (Dietzschold et al. 1990) and identified the immunocritical amino acids of the G5 epitope (van der Heijden et al. 1993).

Epitopic peptides have also been used as immunogens to induce protection against the homologous virus. The synthetic peptide (G5-24) that mimics a linear epitope of rabies virus, in combination with a T-helper epitopic peptide, protected immunized mice against a lethal exposure to rabies virus (Dietzschold et al. 1990). Guinea pigs and cattle vaccinated with peptides representing immunogenic regions of the VP1 capsid protein of foot and mouth disease virus (FMDV) were protected from subsequent viral challenge (Bittle et al. 1982, Dimarchi et al. 1986, Steward et al. 1991). Synthetic peptide vaccines are an attractive alternative to the more traditional whole virion vaccines, because they can be strain specific, mass produced inexpensively, have a long shelf-life, and are unable to cause any disease symptoms (Plaue et al. 1990, Arnon & van Regenmortel 1992).

The 3 synthetic peptides used in this experiment each contained 13 aa residues. Each peptide was presented in 2 different forms to rainbow trout. The forms consisted of the peptide conjugated to either a carrier protein or synthesized directly upon a multiple antigenic peptide (MAP) matrix. The purpose of this study was to determine whether any of the peptide constructs, when injected into rainbow trout, could stimulate antibody production. The trout sera were analyzed by ELISA for the presence of antibodies that could bind to the peptides or to intact virions. The sera were also tested by a complement-mediated neutralization assay for the presence of antibodies with the ability to neutralize IHNV. Our results provide information about the humoral immune response of salmonids to synthetic peptides that were designed to mimic the proposed antigenic sites on the glycoprotein of IHNV.

MATERIALS AND METHODS

Viruses and cells. We used the Western Regional Aquaculture Center (WRAC) strain of IHNV (ATCC VR-1392) isolated in 1982 from rainbow trout in the Hagerman Valley of Idaho, USA. The virus was propagated and assayed using the *epithelioma papulosum cyprini* (EPC) cell line (Fijan et al. 1983). Cells were grown at 25°C in minimum essential media (MEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 0.3% tryptose phosphate broth (Difco), 2 mM L-glutamine (Difco), 2.5 µg ml^{-1} fungizone (Gibco), 100 µg ml^{-1} streptomycin, 100 IU ml^{-1} penicillin, and 100 µg ml^{-1} gentamicin sulphate (US Biochem Corp.), and Tris-buffered to pH 7.5.

Viral stocks were cultured on EPC monolayers for 7 d at 15°C. The culture fluid was harvested and centrifuged at 100 000 × *g* for 20 min at 4°C. A subsample of the supernatant was used in a plaque assay (Burke & Mulcahy 1980) to determine virus concentration. Aliquots of the viral supernatant were stored at –80°C and utilized for positive controls, complement-mediated neutralization assays, and ELISAs. For the ELISA, virus was further purified on discontinuous and continuous sucrose gradients (Hsu et al. 1986) and used as a coating antigen.

Peptide sequence selection. The primary structure of the peptides used in this study was based on amino acid sequences that included the epitopes identified by Huang (1993). The choice of a particular amino acid for initiation of peptide synthesis was determined by the hydropathic values of constituent amino acids (Kyte & Doolittle 1982) and by the desire to place the immunoreactive site toward the free amino terminus of the peptide construct. Peptide sequences with higher hydrophilicity were selected because they would be more exposed in solution (Grant 1992), increasing their potential to bind to B-cell ligands. The 3 peptide sequences selected for the experiment, designated P76, P226, and P268, are shown in Fig 1.

Preparation of peptide inocula. An Applied Biosystems Inc. (ABI) 431A solid phase peptide synthesizer and 9-fluorenylmethoxycarbonyl (Fmoc) protected

Peptide[a]	N-terminus——C-terminus[b]
P76	Tyr-Pro-Thr-Ser-Ile-*Arg*-Ser-Leu-Ser-Val-Gly-Asn-Asp
P226	Asp-Lys-Ile-Ser-*Asn-Arg*-Val-Val-Lys-Ala-Thr-Ser-Tyr
P268	Ser-Val-Val-Tyr-*Asn*-Ser-Gly-Ser-*Glu*-Ile-Leu-Ser-Phe

*Delineates immunoreactive sites on the glycoprotein as determined by Huang (1993)

[a]The numbers designate the starting amino acid residue in the peptide (P) based on the primary structure of the entire IHNV glycoprotein (Koener et al. 1987)

[b]Cysteine (Cys) residues were added to the carboxyl terminus of these peptides for conjugation to a BSA carrier molecule

Fig. 1. Peptide primary structure

amino acids were used to produce the peptides. Peptides to be conjugated to the bovine serum albumin (BSA) carrier were synthesized on a *p*-hydroxymethylphenoxymethyl polystyrene (HMP) resin, while 8-branched, multiple antigenic peptides (MAP8) were synthesized directly onto the MAP matrix-resin (ABI). Peptides were cleaved from the resins using methods described by the manufacturer (ABI). Cleavage mixtures and reactions times were sequence-dependent and varied for each peptide preparation. After cleavage from the resins, free peptides were isolated by repeated cycles of centrifugation at 2000 × *g* for 10 min at room temperature and washing in methyl *t*-butyl ether. The final peptide precipitate was dried overnight in a chemical fume hood at room temperature and then stored at −20°C. Peptide purity was verified by reverse phase high performance liquid chromatography (HPLC) on a Waters Delta Pak C-18 column (8 × 100 mm, 15 µm particle, 30 nm pore size) using 0.1% trifluoroacetic acid (TFA) in water and 80% acetonitrile in 0.1% TFA buffers.

Free peptides were conjugated to the BSA carrier protein by binding a sulfhydryl group of a cysteine residue (added to the carboxyl terminus of the peptide during synthesis) to the side chain amino groups of the lysine residues in the BSA carrier. Briefly, 10.0 mg of peptide-Cys (P76-Cys, P226-Cys, or P268-Cys) was dissolved in 0.25 ml of DMSO and phosphate buffered saline (PBS) was added to bring the reaction mixture to 1.0 ml. Activated lyophilized maleimide BSA (Pierce #77115) was added to each peptide-Cys suspension and the peptide-Cys-BSA conjugates were purified by centrifugation in Centricon 30 microconcentrators (Amicon) according to manufacturer specified protocols. Each peptide inoculum (P-Cys-BSA or P-MAP8) was emulsified 1:1 in Freund's complete adjuvant (FCA) by the stepwise addition method (Moncada et al. 1993). An unconjugated preparation of BSA, emulsified in FCA, served as the negative control. A suspension of concentrated IHNV (WRAC strain) killed by the addition of 10% neutral buffered formalin and a live, low virulence strain of IHNV (Clear Springs isolate #184-90) were the positive controls.

Immunization of rainbow trout. Immunization was conducted in the research laboratories of Clear Springs Foods, Inc., Buhl, Idaho, USA. Ten groups of 2-yr-old rainbow trout, each weighing approximately 900 g, were anesthetized with tricaine methane sulfonate (MS-222) and received a 500 µl intraperitoneal injection of one of the following inocula: P76-Cys-BSA, P226-Cys-BSA, P268-Cys-BSA, P76+MAP8, P226+MAP8, P268+MAP8, a mixture of the 3 MAP8 peptides, BSA (− control), and inactivated IHNV or low virulence IHNV (+ controls). Final peptide concentration administered was approximately 1 mg per trout. Negative controls received 1 mg of BSA. Positive controls received 4.13×10^7 PFU formalin inactivated IHNV or 3.14×10^7 PFU of the low virulence IHNV isolate. Prior to injection, a subsample of trout from each tank were anesthetized, bled to obtain a pre-immunization sample (~1 ml), and confirmed negative by the complement-mediated neutralization assay. Each treatment group consisted of 10 Floy-tagged trout randomly distributed among four 1140 l tanks provided with pathogen-free water at 15°C. Ten non-injected control trout with no known history of IHN were also distributed with the other immunized fish. The 2 groups of positive control fish were isolated in separate tanks.

Collection of sera. Immunized trout were held for a total of 8 wk in pathogen-free water at 15°C. At 2 wk intervals, the fish were anesthetized and 1 ml volume of blood was removed via caudal puncture. For the final bleeding (Week 8), fish were sacrificed and the maximum amount of blood was extracted. To obtain sera, the blood samples were allowed to clot overnight at 4°C and were then centrifuged for 5 min in a Damon/IEC Hematocrit MB centrifuge. Sera were stored at −80°C until analysis.

Enzyme linked immunosorbent assay (ELISA). Immulon II 96-well microtiter plates (Dynatech Inc.) were coated with 0.1 µg of purified IHNV or peptide-MAP8 in a carbonate buffer (pH 9.6) for 16 h at 4°C. Plates were rinsed 3 times with PBS containing 0.05% Tween 20 detergent (PBST, Sigma) on an automated microplate washer (Bio-tek). Wells were blocked with 3% BSA in PBST for 1 h at 4°C and rinsed again. Individual trout serum samples were diluted with 1% BSA in PBS in a range from 1:20 to 1:2560 and 100 µl samples of each dilution were added to wells in duplicate and incubated for 16 h at 17°C in a moist chamber. Plates were rinsed with PBST as described previously. One hundred µl of horseradish peroxidase goat anti-trout immunoglobulin (Kirkegaard and Perry) was placed in the sample wells and incubated at room temperature for 1 h. Plates were rinsed 4 times with PBST. One hun-

dred µl of the chromagen, a 1:1 solution of 0.6 g l^{-1} ABTS [2,2' azino-bis (3-ethylbenzthiazoline 6-sulfonic acid)] in a glycine buffer and 0.02% hydrogen peroxide in a citric acid buffer, was added to the wells and allowed to react for 30 min at room temperature in a moist chamber. The reaction was stopped by the addition of 100 µl of 1% sodium dodecyl sulfate (SDS) to each well. Optical densities (OD) of each sample were measured at 405 nm using a spectrophotometer plate reader (Bio-tek). A single dilution, either 1:160 or 1:320 for plates coated with IHNV or the peptides, respectively, was selected to maximize the differences between the mean OD of the negative and positive control sera. A sample was designated positive if its average OD value was greater than 2 standard deviations above the mean of the negative control sera. Positive control sera for the assay consisted of a pool of convalescent sera from rainbow trout surviving an IHNV infection. Negative control sera for the assay consisted of a pool of sera from stock rainbow trout with no known previous exposure to IHNV. Specificity of the ELISA was confirmed by pooled convalescent anti-IHNV sera that bound to each of the 3 peptide constructs (P76+MAP8, P226+MAP8, or P268+MAP8) or to purified IHNV, which were used in coating the ELISA plates.

Complement-mediated neutralization assay. This assay was modified from the complement-dependent neutralization test described by LaPatra et al. (1993). All sera were inactivated by incubation for 30 min at 45°C. A 2-fold dilution series of the sera ranging from 1:20 to 1:160 in Hanks' buffered salt solution (HBSS, Sigma) was performed in a 96-well plate (Costar). Fifty µl of 10^4 PFU ml^{-1} IHNV was added to all wells and incubated for 30 min on a rocker platform at 18°C. The same volume of complement diluted 10-fold in HBSS was then added to all wells and incubated again under the same conditions.

Twenty-four hour old EPC cell monolayers in 48-well plates (Costar #3548) were pretreated with 10 µl per well of 7% polyethylene glycol (Fisher Chemical) for 10 min. Wells were then inoculated with 50 µl of the reaction mixtures in duplicate. Samples were allowed to adsorb to the cells for 1 h at 18°C on a rocker. The inoculum was removed and EPC cells were overlaid with 0.5 ml per well of 0.7% methylcellulose (Sigma) in MEM with 5% fetal bovine serum. Cells were incubated for 7 d at 18°C, then fixed and stained with a 1% crystal violet-formalin reagent for 1 h. Neutralization titers were determined by the reciprocal of the highest serum dilution that produced at least a 50% reduction in the number of plaques relative to negative controls treated with sera pooled from stock rainbow trout with no known exposure to IHNV.

RESULTS

Mortalities incurred after immunization

After immunization, fish were monitored daily for mortalities. In general, mortalities were low for all treatment and control groups. Trout immunized with P76-Cys-BSA had the highest cumulative mortality (30%). Fish injected with P76+MAP8, P226+MAP8, P226-Cys-BSA, and the low virulence (LV)-IHNV had mortalities of 20%. Fish injected with formalin inactivated (FI)-IHNV experienced 10% mortality. All other treatment groups had no mortalities. The majority of the mortalities occurred after Week 6 and appeared to be a result of excessive handling during sampling. When compared with controls, fish inoculated with peptide constructs did not have a significant difference in mortality indicating the peptides themselves had no adverse effect on the treated fish.

Recognition of peptides by anti-peptide sera

When serum samples were collected from each fish at Week 8 and screened by ELISA for the presence of antibodies that bound to homologous and/or heterologous peptides, only a few binding patterns were demonstrated. A significant proportion of the antisera extracted from fish injected with P76+MAP8 or P76-Cys-BSA recognized the homologous peptide (P76) more than the heterologous peptides (P226 and P268) (Table 1). In contrast, antisera from fish inoculated with either of the P226 or the P268 constructs recognized P76 equally well or better than the homologous peptide or the other heterologous peptide. Generally, a low level of peptide recognition was observed for all antisera tested (Table 1).

The immunogenicity of the peptide inocula could not be determined due to the high degree of individual variation within each treatment group. For example, trout inoculated with P76-Cys-BSA had the highest percentage (57%) of positive serum samples that bound to the homologous immunizing peptide (P76) (Table 1). However, this was only slightly greater than the percent (50%) of positive samples from the non-injected, negative control sera that bound to the same peptide (P76). Further, 1 sample of P76-Cys-BSA antisera bound only to the heterologous peptide (P226) and 2 antiserum samples bound to none of the peptides. Antibody binding of the antisera from the other treatment groups was similarly variable (Table 1). Sera from positive control fish injected with intact virus (FI-IHNV or LV-IHNV) or from negative control (BSA injected and non-injected) fish also recognized the 3 peptides to varying degrees (Table 1).

Recognition of native virus by anti-peptide sera

Recognition of native IHNV was low for sera from fish injected with the various peptide constructs. However, positive control sera extracted 6 wk after immunization from fish injected with formalin inactivated (FI-) or low virulence (LV-) IHNV had binding frequencies of 89 and 100%, respectively (Table 2). Among sera from the non-injected (NI) negative control fish, only 1 sample was positive 2 wk post-immunization. All NI serum samples for Weeks 4, 6, and 8 were negative. Binding of negative control sera from BSA-injected fish ranged from 30% at 2 wk post-immunization to 0% by Week 8. Among the test sera, the highest binding rate (33%) was noted 6 wk after injection with P76+MAP8. All other test sera demonstrated binding rates less than, or equal to, 30%.

Table 1. Recognition of peptides by anti-peptide sera. Serum samples from the 8-wk bleed were tested by ELISA against 3 peptide coating antigens (P76+MAP8, P226+MAP8, P268+MAP8). A sample was designated positive (i.e. binding) if its average OD value was greater than 2 standard deviations above the means of the negative control sera OD at a 1:320 dilution. Mean OD and standard deviation of the negative control sera: P76, 0.285 ± 0.034; P226, 0.808 ± 0.103; P268, 0.309 ± 0.046 (n = 6). Number in parentheses represents number of serum samples tested

Treatment group	% positive samples		
	P76	P226	P268
P76+MAP8	50% (8)	12% (8)	25% (8)
P226+MAP8	57% (7)	14% (7)	12% (8)
P268+MAP8	20% (10)	0% (10)	20% (10)
Mixed MAP8	40% (10)	20% (10)	30% (10)
P76-Cys-BSA	57% (7)	14% (7)	0% (7)
P226-Cys-BSA	25% (8)	25% (8)	12% (8)
P268-Cys-BSA	40% (10)	20% (10)	0% (10)
Negative control (BSA)	40% (10)	10% (10)	0% (10)
Negative control (NI)	50% (8)	12% (8)	0% (8)
Positive control (FI-IHNV)	22% (9)	11% (9)	11% (9)
Positive control (LV-IHNV)	12% (8)	12% (8)	0% (8)

Table 2. Recognition of native virus by anti-peptide sera. Bi-weekly serum samples were tested by ELISA against native IHNV. A sample was designated positive if its average OD value was greater than 2 standard deviations above the means of the negative control sera OD at a 1:160 dilution. Mean OD and standard deviation of the negative control sera: 2 wk, 0.276 ± 0.041 (n = 7); 4 wk, 0.176 ± 0.068 (n = 7); 6 wk, 0.182 ± 0.072 (n = 6); 8 wk, 0.308 ± 0.074 (n = 6). Number in parentheses represents number of serum samples tested

Treatment group	% positive samples			
	2 wk	4 wk	6 wk	8 wk
P76+MAP8	0% (10)	30% (10)	33% (9)	25% (8)
P226+MAP8	10% (10)	10% (10)	11% (9)	0% (7)
P268+MAP8	10% (10)	20% (10)	10% (10)	20% (10)
Mixed MAP8	20% (10)	20% (10)	0% (10)	10% (10)
P76-Cys-BSA	10% (10)	10% (10)	0% (9)	0% (7)
P226-Cys-BSA	10% (10)	10% (10)	20% (10)	12% (8)
P268-Cys-BSA	10% (10)	10% (10)	30% (10)	10% (10)
Negative control (BSA)	30% (10)	20% (10)	10% (10)	0% (10)
Negative control (NI)	12% (8)	0% (8)	0% (8)	0% (8)
Positive control (FI-IHNV)	11% (9)	22% (9)	89% (9)	0% (9)
Positive control (LV-IHNV)	44% (9)	11% (9)	100% (8)	50% (8)

Neutralization activity of the anti-peptide sera

All of the sera were tested for neutralization of IHNV using a complement-mediated assay. Among all of the sera from the peptide-injected fish (n = 70), only one, from a fish injected with P268+MAP8, elicited a strong neutralization titer (Table 3). The sera obtained from this fish at Weeks 4 and 8 had titers greater than 160, but sera from Weeks 2 and 6 had titers of 0 and 20, respectively. The serum from this individual also bound to P76, P268 and to native IHNV in the ELISA.

Seventy percent of the LV-IHNV positive control sera had neutralization titers that exceeded 160 at Weeks 4, 6, and 8 (Table 3). Among the 3 fish that did not respond, one (W-8) was lost during the first blood extraction, one (W-13) died immediately after the Week 4 sample was collected, and one (W-11) had consistently low titers. Test sera from both negative controls (NI and BSA) and the other positive control (FI IHNV) groups demonstrated no significant neutralization activity (data not shown).

DISCUSSION

Rainbow trout immunized with various peptide constructs representing putative epitopes on the IHNV glycoprotein produced antisera with little or no ability to recognize either the native virus or the peptides themselves. One explanation for the failure of our peptides to stimulate an immune response could be that the amino acid sequences we chose do not represent epitopes on the glycoprotein of IHNV that are recognized by fish. We selected sequences on the glycoprotein of IHNV based on immunocritical sites identified

Table 3. Neutralization activity of anti-peptide sera. The P268+MAP8 antisera from fish #52 represented the only test sera to demonstrate neutralization activity. The titers of all 10 positive control antisera from fish injected with low virulence (LV)-IHNV are also listed. Neutralization titers are expressed as the reciprocal of the highest dilution that corresponded to at least a 50% reduction in the number of plaques relative to negative control wells. ns: no sample was taken, usually due to mortality

Inoculum treatment	Tag #	Neutralization titer			
		2 wk	4 wk	6 wk	8 wk
P268+MAP8	52	0	>160	20	>160
Positive control (LV-IHNV)	W3	20	>160	>160	>160
Positive control (LV-IHNV)	W4	0	>160	160	>160
Positive control (LV-IHNV)	W5	20	>160	>160	>160
Positive control (LV-IHNV)	W6	40	>160	>160	>160
Positive control (LV-IHNV)	W7	0	>160	>160	>160
Positive control (LV-IHNV)	W8	ns	ns	ns	ns
Positive control (LV-IHNV)	W9	80	>160	160	>160
Positive control (LV-IHNV)	W10	40	>160	160	>160
Positive control (LV-IHNV)	W11	0	40	40	0
Positive control (LV-IHNV)	W13	0	20	ns	ns

by MAbs developed in mice (Huang 1993). Immunogenicity of these mouse-selected epitopes may be low in fish, resulting in little or no antibody production. The proposed epitopes may be immunodominant in mammalian systems, but the more primitive fish immune system may recognize other B-cell epitopes.

Reports in the literature confirm that fish immune cells can recognize different antigenic sites than mammalian lymphocytes. The bacterial fish pathogens *Vibrio anguillarum* serotype 02 and *V. ordalii* were initially thought to both belong to the same serotype 02 group based on Western immunoblots with rabbit polyclonal antiserum (Chart & Trust 1984). Based on this assumption, a bivalent vaccine containing killed preparations of *V. anguillarum* serotype 1 and *V. ordalii* was used to vaccinate salmonid smolts (Smith 1988). Subsequent outbreaks of *V. anguillarum* serotype 02 in these fish brought into question the effectiveness of *V. ordalii* in cross-protecting fish against other serotype 02 strains. The antigenic properties of the *Vibrio* lipopolysaccharide (LPS) were examined by Mutharia et al. (1993) using antisera produced in both rabbits and fish. Rabbits immunized with either of the *Vibrio* strains produced antibodies that recognized both common and strain-specific epitopes. When these bacteria were injected into fish, common and strain-specific epitopes were also recognized by fish lymphocytes, but these epitopes were different from the antigenic sites recognized by rabbits. Furthermore, protection of fish relied on those strain-specific epitopes uniquely recognized by trout.

Two studies have also tested synthetic peptides as immunogens in a fish host. Mourich & Leong (1991) injected 5 overlapping synthetic peptides, spanning aa 274–340 on the IHNV glycoprotein, into mice and rainbow trout. All 5 of the mouse generated antisera bound to the glycoprotein, whereas only 1 peptide (aa 321–340) induced the production of trout antisera that reacted with the IHNV glycoprotein. In another report, a cytotoxic T-cell (Tc) epitope on the nucleoprotein of vesicular stomatitis virus (VSV), a mammalian rhabdovirus having a high sequence homology with a region on the nucleoprotein of the fish rhabdovirus, viral hemorrhagic septicemia virus (VHSV), was evaluated for its ability to cross protect (Estepa & Coll 1993). The immunodominance of this VSV Tc epitope was established in a mouse model. When rainbow trout were immunized with peptides mimicking this Tc epitope and subsequently challenged with VHSV, mortality was significantly greater in immunized fish compared to controls. These examples emphasize the potential problems of studying the antigenicity or immunogenicity of suspected epitopes on fish pathogens using serological data from non-host species.

If the epitopes on the glycoprotein of IHNV identified by Huang (1993) can be recognized by the trout immune system, an alternative explanation for the low immune response to the peptides is necessary. The lack of immunogenicity for some of the peptides was not entirely unexpected due to their chemical nature. The amino acids in P76 had a net charge of zero and were difficult to dissolve. This was especially crucial when P76 was coupled to the BSA carrier, because successful conjugation was dependent on the peptide remaining in solution. In addition, the hydropathy plot of the IHNV glycoprotein (Koener et al. 1987) revealed that this region was relatively neutral and most epitopes tend to be located in hydrophilic regions (Hopp and Woods 1981). However, the amino acids of P76 had a total hydropathic value of −5.8, with a greater negative value corresponding to greater hydrophilicity (Kyte & Doolittle 1982), which should have increased its biological activity.

The immunogenicity of peptide-226 (P226) may have been poor because the epitope is conformation dependent. Secondly, the enzymatic stability of this peptide was low (Grant 1992) because the 2 Lys-X and 1 Arg-X amide bonds were susceptible to trypsin-like enzyme degradation and the N-terminal Phe residue linkage can be reduced by chymotrypsin-like enzymes (Fig. 1). This proteolytic activity may have decreased the serum half-life of the peptide, reducing its interaction with the trout immune system.

One of the immunocritical sites (aa 272) on P268 is believed to be linear (Huang 1993) and is located in a hydrophilic region on the IHNV glycoprotein. For these reasons P268 was expected to be highly immunogenic. This peptide was predicted to be fairly resistant to proteolysis, but the C-terminal Phe-X linkage to the carrier molecule was susceptible to chymotrypsin-like enzyme activity. Smaller peptides are not able to maintain stable secondary structures (Cornette et al. 1989, Wang et al. 1993), but they can bend. The presence of a glycine (Gly) residue in P268 indicates a possible bend and this could have decreased the efficient recognition of the epitopic peptides by B-cells (Grant 1992). Another consideration is that the total hydropathic value of P268 was +7.0, which is slightly hydrophobic and may have lowered the biological activity of the peptide in solution.

A third explanation for the low immunogenicity of the peptides might be the lack of T-cell stimulation. The peptides in this study are most likely B-cell epitopic homologs (Huang 1993); however, both T-helper cell and B-cell epitopic peptides may be required to mount a sufficient immune response in rainbow trout. Classically, large carrier proteins like BSA are used to increase the antigenic mass of an immunogen and provide T-helper cell stimulation; however, MAP8 carrier molecules are considered immunogenically inert (Tam 1988) and would provide no T-cell stimulus. Vallejo et al. (1992) stress that determining the T- and B-cell specificities and effector functions (e.g. proliferative responses, interleukin secretion and cytotoxic activities) of T-cells in fish are essential to vaccine development.

The importance of T-cell activity in the immune response against VHSV has been demonstrated by Estepa et al. (1991). Adherent cells, taken from a mixed culture of kidney cells from immunized trout, were stimulated by both VHSV G and N-proteins, but the adherent cells had a greater response to the N-protein than the G. The authors felt that the N-protein stimulates immunological memory in these immunized cells *in vitro* as has been previously demonstrated for VSV (Puddington et al. 1986) and rabies viruses (Dietzschold et al. 1987). Further, the N-protein was shown to augment the immune response of fish against IHNV (Oberg et al. 1991, Anderson et al. 1996). It was suggested that the N-protein may stimulate T-helper or T-cytotoxic activity.

Although some trout antisera did exhibit binding activity against the peptides, or native virus, there was a high degree of variation among fish. Most disturbing was the apparent presence of natural antibodies in a few non-injected (NI) negative control fish (Tables 1 & 2). This variability in antibody binding by fish from the same treatment groups may be a result of distinct characteristics present in individual fish that influence their immune response to antigens. These characteristics could include genetic heterogeneity, the presence of natural antibodies, or previous exposure to homologous or cross-reactive heterologous antigens.

Individual variation in immune response due to genetic heterogeneity has been demonstrated in fish (Dorson 1984). Five *Renibacterium salmoninarum* bacterins were evaluated by Sakai et al. (1993), who found that some rainbow trout within the different treatment groups had high agglutinating antibody titers, whereas other trout receiving the same bacterin had no detectable antibody titer. Van Ginkel et al. (1992) characterized anti-hapten antibodies produced *in vitro* by channel catfish *Ictalurus punctatus* peripheral blood lymphocytes (PBL) and compared them to antibodies produced *in vivo* by the same fish. They observed that the relative quantity of antibodies produced *in vitro* was equivalent to the number present in that same fish's serum and proposed that genetic predisposition may be responsible for determining the quantity of hapten-specific PBLs in a fish's bloodstream. Individual variation in the proliferation of leucocyctes harvested from rainbow trout that have survived a VHSV infection has been demonstrated. Synthetic peptides representing the G-protein of VHSV were tested *in vitro* for their ability to stimulate proliferation of rainbow trout kidney leucocytes (Lorenzo et al. 1995). The peptides only stimulated proliferation of leucocytes harvested from survivors of a VHSV infection. Further, the degree of stimulation varied greatly between survivors in both magnitude and type of peptide used.

The use of fish with different major histocompability complex (MHC) haplotypes may explain, in part, the variation seen in antibody binding. Fish T-cell epitopes are presented in conjunction with MHC molecules in order for immunostimulation to occur (Vallejo et al. 1992). The BSA carrier may have provided T-cell epitopes that were only recognized by certain antigen presenting cells (APC) expressing a specific MHC. In our study, only those fish that possessed the appropriate APC could process these T-cell epitopes, link them to the MHC at the cell surface, and then present them to B-lymphocytes. Development of a truly inbred strain of rainbow trout with a defined MHC would eliminate the ambiguity in evaluating immunogenicity of peptides, due to the genetic homogeneity of the host species. However, this would not facilitate finding the most efficacious vaccine preparations for use in the fisheries industry, where broad genetic diversity is typical and desirable.

Fish to fish variation in immune responsiveness may also be due to natural antibodies, or to previous exposure to homologous or cross-reactive heterologous

antigens. Fuda et al. (1991) measured serum antibody levels in developing masu salmon *Oncorhynchus masou* and noted a positive correlation between body weight and immunoglobulin concentrations (with the exception of smolts) for fish up to 489 d old. The investigators hypothesized that the presence of natural antibodies varies between individual fish and could be due to the normal physiological production of immunoglobulins without antigenic stimulation. Another hypothesis is that a fish immune system is exposed to a variety of external antigens during its lifetime, resulting in 'natural' antibody production (Ingram 1980). These antibodies, which may exhibit cross-reactive binding activity due to low binding specificity, have been detected in rainbow trout (Olesen & Jørgensen 1986).

Trout used in this experiment were 2 yr old and probably exposed to a variety of antigens prior to immunization. As a result, these older fish may have a greater amount of natural serum antibodies, which could explain the presence of binding antibodies among some trout within certain treatment groups, and from the non-injected and BSA control groups. Only one fish injected with P268+MAP8 produced antisera which neutralized IHNV (Table 3). It seems doubtful that this neutralization activity resulted from an immune response induced by the peptide, since no other antisera from any treatment group exhibited any significant neutralization activity. While unlikely, this fish may have survived an subclinical IHNV infection that was never detected and as a result had a very high neutralization titer (Table 3). The lack of neutralization by the other antisera having some binding activity may be due to a low number of antibodies present. Flamand et al. (1993) determined the number of immunoglobulin (IgG or IgM) molecules required for neutralization of rabies virus at 3 primary antigenic sites. At antibody binding levels up to 130 IgG or 30 IgM molecules per virion, infectivity was still preserved. This may explain why, in our experiment, binding antibodies against IHNV could be present, but neutralization was absent. The peptides may have stimulated the production of antibodies, but at concentrations below the neutralizing dose.

Our results demonstrate the need for a greater fundamental understanding of fish immune mechanisms in order to find an effective means of preventing IHN. Determining the true immunodominant B- and T-cell epitopes of IHNV that are recognized by fish possessing a range of MHC haplotypes are critical initial steps. The types of non-specific immunostimuli (i.e. adjuvants and immunostimulants) required for enhancement of the immune response in a particular fish species also need to be assessed. Determining the critical components involved in eliciting an immune response will not only increase our overall understanding of the fish immune system, but will provide us with the information needed to design a successful vaccine for control IHN.

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