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Title: VARIANTS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS
SELECTED WITH GLYCOPROTEIN-SPECIFIC MONOCLONAL ANTIBODIES

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The development of an effective infectious hematopoietic necrosis virus (IHNV) vaccine depends on a better understanding of the antigenic structure and variation among isolates. Three objectives to elucidate this were: (1) to generate a panel of antigenic derivatives of the IHNV glycoprotein by monoclonal antibody-mediated selection of neutralization-resistant variant viruses, (2) to determine if mutation in a critical site for viral neutralization affects viral virulence in vivo and characteristics in vitro, and (3) to establish an antigenic relationship between the Round Butte (RB) and 193-110 strains of IHNV.

A single anti-glycoprotein monoclonal antibody, RB/B5, was used to select neutralization-resistant variants of the RB and 193-110 strains of IHNV. The virulence of the battery of IHNV variants was tested in rainbow trout by waterborne challenge. Two of these

variants, RB-1 and 193-110-4, exhibited decreased virulence for the host. Vaccination with RB-1 and 193-110-4 conferred protection to rainbow trout challenged with wild-type virus. Interference did not appear to be the mechanism for protection.

In vitro, variants RB-1 and 193-110-4 retained several wild-type characteristics. Titers were normal, differences among the structural proteins were not detected, variants were not temperature-sensitive mutants, and variants were neutralized by hyperimmune serum. However, variant replication was slower than wild-type virus at permissive temperatures and as a result viral plaque size was smaller. Experiments with neutralizing and nonneutralizing monoclonal antibodies indicated that the RB and 193-110 strains have overlapping neutralization epitopes and share conserved sequences at a similar region on the IHNV glycoprotein.

Variants of Infectious Hematopoietic Necrosis Virus
Selected with Glycoprotein-specific Monoclonal Antibodies

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VARIANTS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS SELECTED WITH GLYCOPROTEIN-SPECIFIC MONOCLONAL ANTIBODIES

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a pathogen of commercially and hatchery reared salmonids. Animals up to two months are the most susceptible and mortality may be excessive (Pilcher and Fryer, 1980). No vaccine nor prophylactic treatments exist and avoidance is the only method of control available. Therefore, development of an effective immunizing agent against IHNV is attractive. Without an appropriate understanding of the antigenic variation among strains of IHNV, there exists little logical basis for the selection of vaccine strains or viral protein components.

The glycoprotein of IHNV is the major immunogenic subunit responsible for the induction of a virus neutralizing antibody. Serological differences among virus strains are not readily detected with polyvalent antiserum but are detected with monoclonal antibodies. The latter reagents can detect small changes in complex macromolecules of organisms and have been used to enrich for neutralization-resistant viruses *in vitro*. These virus variants can be used as markers for individual epitopes and an epitope map can be constructed based on the differential reactivity of monoclonal antibodies with

these variants. In addition, virus-host interactions can be studied using variant viruses. The objectives of this study were to: (1) generate a panel of antigenic derivatives of the IHNV glycoprotein by monoclonal antibody-mediated selection of neutralization-resistant variant viruses, (2) to determine if mutation in a critical site for viral neutralization affects viral virulence in vivo and characteristics in vitro, and (3) to establish an antigenic relationship between the Round Butte (RB) and 193-110 strains of IHNV.

In this study, it was found that neutralization-resistant variants retained several wild-type characteristics; although, variants exhibited slower rates of replication. Analysis of RB and 193-110 variants suggested both share a common epitope important for neutralization. Two variants had reduced virulence for fish and one variant had variable virulence in three stocks of rainbow trout. The protective response in the host was not a result of autointerference.

LITERATURE REVIEW

Infectious Hematopoietic Necrosis Virus

Infectious hematopoietic necrosis virus (IHNV) is responsible for explosive epizootics in commercially and hatchery-reared salmonids of the Pacific Northwest, Alaska, British Columbia, and Japan. Mortality is highest among small fish, particularly <2 gm in size. This virus can be detected in animals undergoing epizootics or in the sex fluids and tissues of mature spawning adults. Survivors of outbreaks are believed to become lifelong carriers; however, viable virus has not been isolated during latent stages (Amend, 1975). Transmission of the virus occurs horizontally (Wingfield and Chan, 1970; Mulcahy et al., 1983) and may occur vertically from the infected adult to progeny (Mulcahy and Pascho, 1985). Avoidance of the virus, destruction of infected fish stocks, and disinfection of contaminated eggs are the only currently available methods of control. Development of an effective vaccine has been hindered by insufficient knowledge of the immune system of small fish, development of an effective immunogen, and a poor understanding of species or stock susceptibilities to different strains of IHNV.

Infectious hematopoietic necrosis virus, a rhabdovirus, is unique in that it produces six, rather than five, structural proteins (Kurath and Leong, 1985).

These proteins include the viral polymerase (L), a glycoprotein (G), two matrix proteins (M_1 and M_2), a nucleocapsid protein (N), and a nonvirion protein (NV). Variation in the molecular weight of the N and G proteins has been observed among strains of IHNV and used as a method to separate these viruses into at least 4 types (Hsu et al., 1986). Strains of IHNV exhibit differences in virulence, species specificity (M. Chen, Ph.D thesis, Oregon State University; Scott LaPatra, personal communication), and growth characteristics (Mulcahy et al., 1984). Differences in the glycoprotein among IHNV isolates which could not be detected with polyvalent antisera (Hsu and Leong, 1985) can be detected with G-specific monoclonal antibodies (C. Arakawa et al., 1986).

Much of the available knowledge concerning IHNV has been acquired since 1951 and has been reviewed extensively by McAllister (1979) and Pilcher and Fryer (1980). Therefore, the review presented here will concentrate on those aspects of the selection and characterization of variants of other viral agents.

Variants Selected with Monoclonal Antibodies

Antibodies directed against viruses are important reagents for studying these agents and their pathogenesis. Early work on characterization of viruses employed polyspecific antisera composed of heterogeneous

populations of antibodies. When the antigenic site recognized by one of the antibodies was altered, the change often went undetected because different areas on the antigen continued to react with antibodies of other specificities. The lack of defined specificity was overcome when Kohler and Milstein (1975) described a method to routinely produce monospecific antibodies in vitro.

Monoclonal antibodies detect subtle changes in the sequence or conformation of a viral antigenic site and make it possible to select neutralization-resistant viruses. Variant and mutant viruses are phenotypically different from wild-type viruses, but only mutant viruses are genotypically defined. Recent studies of variants have revealed valuable information about virion architecture, mechanisms of neutralization, antigenic relationships among viruses, virus-host interactions, and viral attachment to cellular receptors (Pollack et al., 1984; Yewdell and Gerhard, 1981). Some of the work with specific viral agents is reported here.

RNA Viruses

Rhabdoviridae

Work with rabies virus illustrates the power of monoclonal antibodies to demonstrate minor antigenic differences among closely related proteins. Definite

antigenic differences among field and vaccine strains, previously considered to be closely related, have been delineated with monoclonal anti-nucleocapsid and anti-glycoprotein antibodies (Wiktor and Koprowski, 1978; 1980; Flamand et al., 1980a and b). These antigenic differences may provide an explanation for some of the failures of vaccines to protect against infection by strains found in the environment.

In an attempt to understand the basis for strain variation and virulence, glycoprotein-specific monoclonal antibodies have been used to select nonneutralized antigenic variants of the Pasteur-derived CVS strain both in vitro (Wiktor and Koprowski, 1980; Coulon et al., 1982) and in vivo (Lafon et al., 1983). Coulon et al. (1982) found these variants retain several wild-type characteristics and they detected no differences in structural proteins on SDS polyacrylamide gels. In addition, monoclonal antibody to nucleocapsid protein recognized both wild-type and variant viruses. When replicated in vitro, wild-type and variant titers were comparable, as was growth at different temperatures. However in contrast to these findings, Dietzschold et al. (1984) found, that in some cases, the electrophoretic mobility of CVS variants was altered and could be attributed to single amino acid changes at glycosylation sites on the glycoprotein.

To demonstrate the protection provided by the immune responses induced by avirulent CVS variants, adult mice were vaccinated by intercerebral injection and foxes received the variants orally. In both instances, animals responded well and were protected upon challenge with parent CVS (Coulon et al., 1983; Flamand et al., 1984). In addition, 226 animals from eight nontarget species, representing the families Microtidae, Muridae, and Mustelidae resisted challenge with variant virus and only Arvicola terrestris displayed signs of infection to oral administration of this same virus (Flamand et al., 1984). Variants were remarkable inducers of early interferon and cytotoxic cells which may explain their lack of pathogenicity for adult mice. Avirulent/attenuated variants retained pathogenicity for suckling mice because this animal has a limited immunological repertoire. Likewise, animals treated with cyclophosphamide, an immunosuppressive drug, exhibited similar susceptibility.

Parent and avirulent variant viruses were compared to detect differences in cell and tissue specificity arising from mutations affecting the site of virulence. In a single study, Reagan and Wunner (1984) explored altered tissue tropism in vitro and reported the ability of a nonpathogenic variant (ERA) to compete for the same cellular receptor site as the parent strain. Therefore, altered cellular receptor specificity did not appear to

represent the mechanism for attenuation. The replication of rabies virus in vivo is largely confined to nerve tissue and causes a wide variety of central nervous signs in warm-blooded animals. In vivo studies by Dietzschold et al., (1985) revealed the distribution of infected neurons in the brain to be the same for both virulent and avirulent CVS, but the rate of viral spread, number of infected neurons, and degree of cellular necrosis was considerably lower in hosts infected with avirulent variants. Kucera et al., (1985) also found that, like strain CVS, variants penetrated the brain, but the infection was slow and involved different cerebral structures. However, variants failed to invade intraocular parasymphathetic oculomotor and retinopetal fibers, but could invade the lens of rats; each characteristic different from virulent CVS. Infections by variants completely subsided by three weeks, presumably because of an efficient host immune response. The importance of an immune response to slow variant infection was demonstrated when addition of immune serum to infected neuroblastoma cells prevented cell-to-cell spread of variants. Normally, rabies virus infection spreads cell to cell in vitro despite continuous addition of anti-rabies serum.

To locate the antigenic sites associated with viral virulence, epitope analysis of variants was performed.

Analysis of CVS variants by Lafon et al. (1983) revealed three functionally independent antigenic sites: two (II and III) which lie in close proximity. Others have proposed the presence of at least four antigenic sites on CVS (Dietzschold et al., 1983) and five on the ERA glycoprotein (Wunner et al., 1984). Coulon et al. (1983) postulated the actual site of CVS virulence to lie within site no. III. It was subsequently shown that pathogenic variants lacked mutations at site III but did have mutations at sites II and IV (Dietzschold et al., 1983). Characterization of the antigenic determinant responsible for pathogenicity revealed a single amino acid change at position 333 of the glycoprotein molecule (Dietzschold et al., 1983; Seif et al., 1984; 1985). Location of this mutation was at base 1,062 in the nucleotide sequence and consisted of a G to A transition. This single substitution resulted in arginine being replaced with either isoleucine in the ERA strain or glutamine in the CVS strain. In addition, amino acid changes were the result of mutations found at bases 1,061 (CVS), 1,072, and 1,133 (ERA) (Seif et al., 1984; Wunner et al., 1984).

Another rhabdovirus, vesicular stomatitis virus (VSV), is a member of the genus Vesiculovirus. This agent has been used extensively to study rhabdoviruses because of its self-limiting infection in humans. This group is

comprised of seven serologically related, but distinct viruses. Variants of two of these serotypes, Indiana (VSV-Ind) and New Jersey, have been selected with neutralizing monoclonal antibodies and, like rabies, four antigenic determinants were delineated (Lefrancois and Lyles, 1983). From these assays, the four antigenic sites of VSV-Ind were determined to be partially overlapping and those of the VSV-NJ sites distinct.

Reoviridae

Mammalian reoviruses, members of the genus Orthoreovirus, consist of three serotypes (types 1, 2, 3) whose specificities are a property of the viral hemagglutinin $\sigma 1$ polypeptide. This protein is encoded in the S1 double-stranded RNA segment of the virus and is responsible for cell and tissue tropism, humoral and cellular immune specificity, hemagglutination, and typically causes fatal encephalitis in mice. Although pathogenesis of orthoreoviruses in human infections has yet to be established, mice provide a model system for studying virulence factors and host-virus interactions. To examine the function of the hemagglutinin (HA) in pathogenesis, a single anti-HA monoclonal antibody was used to select antigenic variants of reovirus type 3 (Dearing Strain) (Spriggs and Fields, 1982). Variant viruses were at least 10^4 times less virulent than the

Dearing strain for suckling mice. Although variants grew to high titers in L cells, when injected intracerebrally, they had decreased virulence in vivo resulting from reduced growth in brain neural tissue. Variant virus infected areas in the brain restricted to the limbic system which includes the hippocampus, hypothalamus, mammillary bodies, and septum; but, the parent strain produced diffuse destruction in the cortex and brain stem (Spriggs et al., 1983; Fields et al., 1984). Variants also have altered capacity to infect the brain following peripheral inoculation. In addition, lack of variant infectivity has been attributed to the efficient production of neutralizing antibodies and cytolytic T-lymphocytes.

The location of the attenuating mutations on reovirus type 3 variants was determined by RNA-sequence analysis. Four of the variants had changes in codon 419 and a fifth variant had a change at codon 340. All of the amino acid substitutions were found on the S1 ds-RNA segment (Bassel-Duby et al., 1986).

Orthomyxoviridae

Influenza A viruses, representatives of the Orthomyxoviridae, exhibit unusual capacity for antigenic modification of their neuraminidase and haemagglutinin (HA) molecules. Major antigenic changes (antigenic shift)

result from large genetic exchanges between influenza viruses. Minor antigenic changes (antigenic drift) are the result of a gradual accumulation of point mutations in the HA followed by selection of mutants by the host immune system (Yewdell et al., 1979). Mechanisms for antigenic drift have been explored by selecting and analyzing variants using monoclonal antibodies. In one of the first studies, Gerhard et al. (1981) analyzed 34 mutant viruses and was able to define four antigenic determinants (Sa, Sb, Ca, Cb) on the HA molecule of the A/PR/8/34 strain (H1N1) which were later expanded by dividing the Ca site into Ca₁ and Ca₂ (Caton et al., 1982). Of these four sites, Sa and Sb were strain specific and largely the targets for antigenic drift.

Variants of the Hong Kong isolate, A/MEM/1/71 (H3N2), had single amino acid changes in the N-terminal half of the HA1 molecule but none in the HA2 (Laver et al., 1979). Three antigenic sites were delineated by monoclonal antibodies (Webster and Laver, 1980) and four using 3-dimensional structure analysis of the HA (Wiley et al., 1981). Under most conditions, mutations of variants developed no new antigenic sites although one variant, MEM/23/84, produced a determinant unique from the wild-type virus. This isolate was used to produce monoclonal antibodies which were used to select second generation variants some of which regained wild-type antigenicity

(Webster et al., 1983).

Unlike A/PR/8/74 and A/MEM/1/71, variants of A/USSR/90/77 (H1N1) exhibited only three antigenic determinants. When sequenced, single mutations could be detected at amino acid 125 or 190, whereas double mutations mapped to residues 125 and 189 (Nakajima et al., 1983; Kendal et al., 1984).

Natali et al. (1983) selected variants of A/Texas/77 (H3N2) which were reacted with polyclonal sera from immune adults and children. Twenty to 41% of adult and 37-58% of children's sera failed to neutralize these variants selected in vitro with monoclonal antibodies. This suggested certain human sera and particularly that of children possess a limited antibody repertoire to the influenza HA and this may provide a mechanism for appearances of new strains in the environment.

Relatively little has been accomplished to select neuraminidase (NA) variants because NA involvement in immunological neutralization and antigenic drift is limited. But, variants of the A/Tokyo/3/67 (H2N2) strain have been selected and it was shown that three, possibly four, nonoverlapping epitopes exist on this molecule (Webster et al., 1982). Five of the seven variants had a single amino acid substitution at residue 344, while another had a substitution which occurred at amino acid 368 (Lentz et al., 1984; Laver et al., 1982).

Influenza type B has been a target for research because of its association with Reye's syndrome, a disease particularly of children. Type B epidemics occur only in humans and are less frequent than those caused by type A. This is a consequence of lower frequencies of, and longer intervals between, antigenic changes. By selecting ten variants of the B/Hong Kong/8/73 which had changes in the hemagglutinin, Webster and Berton (1981) explored the mechanism for antigenic drift of type B and were able to define three partially overlapping determinants. Six of the variants which they studied were distinguishable from parent virus with immune ferret sera. These results indicated the epidemiological potential of variants because neutralization-resistant viruses in the host are believed to give rise to new strains. However, it has been demonstrated that variant selection frequencies *in vitro* were 1 in 10^8 ; therefore, the probability of these viruses appearing in nature would be extremely low (Lubeck et al., 1980; Webster and Berton, 1981).

Influenza B variants have a high incidence of multiple nucleotide substitutions which, on occasion, result in double amino acid changes (Hovanec and Air, 1984). This high incidence of mutations with low selection frequencies could not be correlated because HA variants of A/USSR/90/77 with multiple changes were isolated at higher frequencies (Nakajima et al., 1983).

Variants of B/OR/5/80 were shown to have epitopes similar to B/Hong Kong/8/83 with an additional two epitopes of unknown biological significance (Berton and Webster., 1985). A total of sixteen different amino acid substitutions at nine positions were detected for the group of variants sequenced (Berton et al., 1984).

Picornaviridae

Despite the development of the inactivated and attenuated poliovirus vaccines in the 1950's and 1960's, little was known about the antigenic structure and location of epitopes responsible for poliovirus neutralization. Poliovirus exists as three antigenically distinct serotypes of which types 1 (Mahony strain) and 3 (P3-Leon-USA-1937) are responsible for the majority of picornavirus-related paralytic disease. The antigens of these two serotypes have been studied extensively using monoclonal antibodies to select neutralization-resistant variants. It was shown that type 3 variant neutralization epitopes cluster into a single antigenic determinant (Minor et al., 1983) and only a limited number of mutations in this region result in neutralization resistance (Evans et al., 1983). These mutations mapped to amino acids 97-103 of the VP1 capsid protein (Minor et al., 1983). In an unusual case, a mutation of Sabin type 3 at base 472 in the nucleotide sequence which occurred

during passage through the human gastrointestinal tract led to increased virulence (Evans et al., 1985). The mutation resulted in the formation of two additional stem-and-loop structures in the RNA secondary structure.

Unlike poliovirus type 3, mutations found in poliovirus type 1 mapped downstream on VP1 at amino acids 221-223, or in the VP2 and VP3 capsid proteins (Diamond et al., 1985). Therefore, mutations mapped distantly in the linear sequence and the six type 1 antigenic determinants represented conformational epitopes (Emini et al., 1983; Crainic et al., 1983). In addition, Diamond et al. (1985) noted that neutralizing monoclonal antibodies retained the ability to bind some poliovirus type 1 variants.

Rhinoviruses comprise the largest genus in the picornavirus family and are best known as the etiological agents of the common cold. Human rhinoviruses constitute several serotypes and monoclonal antibodies have been used to relate rhinovirus antigens to poliovirus antigens. Variants of human rhinovirus 14 were selected with neutralizing monoclonal antibodies and epitopes were found on VP1 (two), VP2 (one), and VP3 (one) (Rossman et al., 1985; Sherry and Rueckert, 1985; Sherry et al., 1986). In some instances, mutations on VP1 and VP3 produced charge alterations which could be detected by isoelectric focusing, suggesting the importance of ionic bond interactions between antigen and neutralizing antibodies.

Paramyxoviridae

In recent years, viruses of the family Paramyxoviridae have been separated from what today is known as the orthomyxoviruses. Paramyxoviruses are unique because their genomes lack segmentation and the hemagglutinin and neuraminidase molecules are located on the same glycoprotein spike. But like influenza viruses, this structure plays a critical role in stimulating host defenses; therefore, it is of great interest antigenically. Two members of the genus Paramyxoviruses have been subjected to immunoselection using monoclonal antibodies: parainfluenza type 1 and mumps virus. Parainfluenza type 1 (6/94 strain), the cause of croup in children, was shown to have four distinct antigenic sites, three of which were nonoverlapping (Yewdell and Gerhard, 1982). In a similar study, mumps virus (Kilham strain) was shown to have only one antigenic determinant because variants were selected with a single monoclonal antibody. However, Love et al. (1985) were able to demonstrate that variants of mumps virus infiltrated fewer neurons and the quantity of viable virus present in the brain was reduced.

Members of the genus Morbillivirus differ from the paramyxoviruses because they possess no neuraminidase. One member of this group, measles (Edmonston strain), was considered to be antigenically stable, but by using immunoselection to isolate three variants, variation was

shown to occur (Birrer et al., 1981). One of these variants was antigenically similar to the street virus Enders 1677, suggesting variation of isolates arising in nature.

DNA Viruses

Herpesviridae

Herpes simplex viruses (HSV) infect only humans and are found throughout the world. The virus encodes four distinct glycoproteins, gE, gC, gB, and gD; the latter three are the primary immunogens which induce neutralizing antibodies. Antibodies to gC are type specific; whereas, those to gB and gD are cross reactive. Because of the prevalence of HSV and persistence in the human population, the immunobiology of this virus has been studied. Holland et al. (1983) selected HSV type 1 variants with either anti-gC or gB monoclonal antibodies. At least two of the gC mutants failed to express the gC molecule in any form while at least two antigenic sites could be delineated on others. To date, herpes represents the only DNA virus from which variants have been successfully selected using neutralizing monoclonal antibodies (Holland et al., 1983). Unlike RNA, DNA has low mutation frequencies because several proofreading enzymes exist to insure fidelity during replication. It has been suggested the rate of evolution for RNA genomes exceeds that for DNA (Holland et

al., 1982) and this may account for the scarcity of DNA variant literature.

MATERIALS AND METHODS

Cell Lines

The chinook salmon (Oncorhynchus tshawytscha) embryo, CHSE-214 (ATCC CRL 1681; Lannan et al., 1984), and epithelioma papillosum cyprini (Cyprinus carpio), EPC (Tomasec and Fijan, 1971), cell lines were used in this study. All cells were grown at ambient temperature (approximately 22° C) in Eagle's minimum essential medium (MEM) with Earle's salts (Gibco), 200 mM L-glutamine (Sigma), and 10% (MEM-10) fetal bovine serum (Hyclone Laboratories Inc.). The growth medium was adjusted to pH 7.4 with 7.5% sodium bicarbonate. Wolf and Quimby (1976) previously described methods for routine culture of fish cells.

For replicating virus, the serum content of the medium was reduced to 5% (MEM-5) and antibiotics were added (penicillin-streptomycin 10,000 U/ml). Cells were grown as 90% monolayers in plastic culture flasks, 24-well plates, or 96-well microplates (Corning Glass Works), and used as 24-h old cultures.

Virus Strains

Four wild-type IHNV strains were used in this study. The Round Butte (RB) strain was isolated from juvenile steelhead trout (Salmo gairdneri) at Round Butte Fish Hatchery by Dan Mulcahy, Oregon State University. The 193-110 strain of IHNV was isolated from rainbow trout

(Salmo gairdneri) juveniles and the H07 strain from rainbow trout x steelhead fingerlings; both strains were isolated in the Hagerman Valley of Idaho and were kindly provided by Nancy Wood of Rangen Research, Inc. The Sacramento River chinook virus (SRCV) was isolated from juvenile chinook salmon at the California Fish and Game Nimbus Fish Hatchery. Two other fish viruses, infectious pancreatic necrosis virus (VR-299) and viral hemorrhagic septicemia virus (F1 P6 LPP2), were used to test the cross-reactivity of nonneutralizing anti-193-110 monoclonal antibodies.

Preparation of Virus Stocks

Both wild-type IHN stock viruses, RB and 193-110, were propagated in CHSE-214 cells grown in 150 cm² flasks. Viruses were inoculated at low multiplicities of infection (MOI = 0.01) and incubated at 18° C for seven days or until extensive cytopathic effect (CPE) was evident. The virus-containing medium was harvested and cellular debris removed by low speed centrifugation for 20 min at 3400 x g. Aliquots of the supernatant were frozen at -70° C.

Antibodies

Development of Anti-IHNV IgG Monoclonal Antibodies

Hybridomas were produced by a modification of the methods described by Mishell and Shiigi (1980) and Lane (1985). A BALB/c mouse was injected intraperitoneally

(i.p.) twice at 21 day intervals with a 0.1 ml suspension of virus in Hank's balanced salt solution (HBSS). Then, four days prior to spleen removal, an additional 0.1 ml booster was administered i.p. Spleen cells of the immunized mouse were fused with Sp/2 myeloma cells in the presence of 50% polyethylene glycol (PEG) 1500 and 400 (4:1). Because mammalian cells were employed as fusion partners, all aqueous solutions used were warmed to 37° C. The excised spleen was teased apart in a Petri dish filled with 5-10 ml of serum-free RPMI medium (Sigma). Equal volumes of the solution were added to two conical tubes for 5 min to settle out the large spleen tissue. The suspensions were transferred to 50 ml tubes containing equal volumes of Sp/2 cell culture fluid and centrifuged for 5 min at 150 x g. To the resulting pellet, 1 ml of PEG solution was added over a 1 min period. This step was repeated twice. Then 7 ml of RPMI, supplemented with 15% fetal bovine serum (FBS), was added over 2 min. The remaining volume of the tube was filled with RPMI, 15% FBS and centrifuged 5 min at 150 x g. The pellets were resuspended in hypoxanthine, aminopterin, and thymidine (HAT) selective medium and plated in 96-well microplates. Hybridomas were screened for IgG production by microneutralization and by enzyme-linked immunosorbent assay (ELISA), using a modification of the methods described by Campbell (1984). Selected hybridomas were

cloned twice by limiting dilution in the presence of mouse spleen feeder cells. The clones were then expanded to 75 cm² flasks for extensive production of monoclonal antibody (MoAb).

Isotype Determination of Monoclonal Antibodies

Monoclonal antibody class and subclass were determined by ELISA. Assay materials were obtained as a commercial mouse monoclonal subisotyping kit (Hyclone Laboratories Inc.) and used according to manufacturer specifications.

Neutralizing Monoclonal and Polyclonal Antibodies

Neutralizing monoclonal antibodies, RB/B5, RB/A3, and 193-110/B4, developed against the RB and 193-110 strains, were provided by Cindy Arakawa, Oregon State University. Rabbit antiserum made against purified Round Butte IHNV was provided by Scott LaPatra, Oregon State University.

Monoclonal IgM Concentration and Partial-purification

The anti-IHNV mouse monoclonal IgM required purification and concentration before it could be used for Western blot analysis. Cellular debris was removed from hybridoma fluid by low speed centrifugation for 15 min at 850 x g. The antibody was concentrated from 200 ml of hybridoma fluid by ultrafiltration (Amicon) over a 300,000 molecular weight exclusion size membrane filter. The antibody-containing fluid was concentrated to a 15 ml

volume and the antibody precipitated with an equal volume of saturated ammonium sulfate at 4° C. The saturated ammonium sulfate was added to the concentrated hybridoma fluid over several minutes while stirring. The sample was then centrifuged 20 min at 850 x g in a TH-4 rotor (Beckman Instruments). The supernatant was decanted and the precipitate resuspended in 0.5 ml phosphate buffered saline (PBS). The sample was then placed into 10 mm dialysis tubing (Spectrum Medical Industries Inc.) and dialyzed 16 h with three changes of PBS to remove the ammonium sulfate.

Selection of Infectious Hematopoietic Necrosis Virus Variants

Wild-type virus stocks replicated in vitro accumulate random mutations with each subsequent passage. Some of these variant viruses lose the ability to be neutralized and can replicate in the presence of neutralizing monoclonal antibody. To select IHNV variants, serial log dilutions of virus stocks were made such that 10^4 to 10^7 infectious units were plated. To prepare an overneutralizing dose of specific MoAb, RB/B5 or RB/A3 were diluted 1:5 with MEM-0 and 0.5 ml of these mixtures aliquoted into each of four capped tubes. The virus dilutions (0.5 ml) were added to both a tube of MEM-0 and a capped tube with MoAb. The contents of the virus + MoAb tube were briefly shaken every 15 min during a 1 h

incubation at room temperature. At the end of this time, 0.1 ml of each the virus + MoAb and virus + MEM-0 dilutions were added to each of six wells in a 96-well microplate containing monolayers of EPC cells. Variants of both wild-type strains were initially selected on a single master microplate and each well showing CPE in the presence of MoAb at the titration endpoint was assumed to represent a different variant. The contents of each well (0.2 ml) were removed after 7 days with a Pasteur pipet and recloned using the methods described above. At each subsequent passage, only wells exhibiting CPE at titration endpoints were used for further clonings. Clonings were continued until the titer of the virus + MoAb equaled the titer of virus + MEM-0. The frequency of variants in wild-type virus stocks was calculated from the master microplate by dividing the virus titer obtained in the presence of antibody by the virus titer obtained in the absence of antibody.

Variants, following selection, were removed from wells showing CPE at the highest dilution and inoculated into 25 cm² flasks containing monolayers of CHSE-214 cells. One milliliter aliquots were frozen at -70° C and used as master stocks. To produce large quantities of variant virus from these stocks, aliquots were treated with hybridoma fluid, diluted 1:5 in MEM-0, for 1 h prior to inoculation. The purpose of this treatment was to

inactivate any back-mutants or residual wild-type virus present and maintain the homogeneity of the variant stock.

Determination of Virus Titer

Titers were determined by the 50% tissue culture infectious dose (TCID₅₀) assay. Serial log dilutions of the virus suspension were made in MEM-0 and a 0.1 ml aliquot of each dilution transferred to each of six wells on a 96-well microplate seeded with CHSE-214 cells. Microplates were sealed with a plastic cover (Dynatech Laboratories Inc.) and incubated at 18° C for 10 days. The number of wells exhibiting CPE were noted and the virus endpoints calculated by the method of Reed and Muench (1938). Viral titers were expressed as TCID₅₀/ml.

Neutralizations

Neutralization tests utilizing monoclonal or polyclonal antibodies were performed in the same manner described for the selection of IHNV variants with the following modifications. Monoclonal antibody 193-110/B4 and a biclonal mixture of 193-110/B4 and RB/B5 or RB/A3 were used to neutralize wild-type and variant viruses. Virus dilutions were incubated with monoclonal antibodies, diluted 1:5 in MEM-0, and with Sp/2 fluid (1:5 in MEM-0) as a control for nonspecific inhibition of viral replication. Virus isolated from experimentally infected fish were confirmed by neutralization tests using

monoclonal antibody RB/B5.

To conduct neutralization tests with rabbit anti-Round Butte serum, 0.5 ml of polyclonal antiserum, diluted 1:16 (MEM-0), was incubated with virus dilutions. Virus + Ab and virus + MEM-0 were added to each of five wells on a 96-well microplate containing monolayers of EPC cells. To each of eight wells, 0.1 ml of diluted, polyclonal antiserum and MEM-0 was added as toxicity and negative controls respectively. Neutralization indices for both monoclonal and polyclonal antibodies were calculated using methods described by Rovozzo and Burke (1973).

Virus Concentration and Partial-purification

Virus-containing medium from lytic cultures was harvested and cellular debris removed by low speed centrifugation for 20 min at 3400 x g. The supernatant was then centrifuged in polyallomer tubes for 1 h at 85,000 x g in an SW 28 rotor (Beckman Instruments). The resulting pellet was resuspended in 0.5 ml of TNE (0.5 M Tris, 0.01 M NaCl, 0.001 M EDTA, pH 7.4) and layered onto a 10-35-50% (W/W) discontinuous sucrose gradient in nitrocellulose tubes. The gradients were centrifuged for 1 h at 120,000 x g in an SW 50.1 rotor (Beckman Instruments). The IHNV band at the 20-35% interface was removed with a probe and peristaltic pump (Auto Densi-flow IIC, Buchler Instruments) and pelleted by centrifugation for 1 h at 120,000 x g in an SW 50.1 rotor. The

partially-purified virus pellet was resuspended in 0.5 ml of TNE or distilled water and frozen at -70° C.

Plaque Size Comparisons

The sizes of the plaques formed in cell monolayers by the variants RB-1 and 193-110-4 were compared with those formed by the homologous wild-type strains using modifications of the methods described by Burke and Mulcahy (1980). Serial log dilutions of virus were prepared in MEM-0 and 0.1 ml of each dilution was added to two wells of a 24-well plate containing monolayer cultures of EPC cells. Following virus adsorption for 1 h on a rocking platform, 0.5 ml of methylcellulose overlay was added to each well. Plates were sealed with a plastic cover and incubated at 18° C for 10 days. Cell sheets were fixed with formalin (37%) for 30 min and stained with crystal violet (1%) for 1 h. The plates were rinsed with water, air dried, and plaque sizes measured with an ocular micrometer fitted onto a dissecting microscope (Wild Inc.). Approximately 200 plaques were measured to the nearest 0.01 mm and averaged.

Effects of Selected Temperatures on Virus Replication

The wild-type strains RB and 193-110 and the variants RB and 193-110-4 were tested for their ability to replicate at various temperatures. The EPC cell line was selected for this experiment because of its ability to

grow at higher temperatures. Monolayer cultures of EPC cells in 24-well plates were infected with 50 TCID₅₀ of virus and 1 ml of MEM-5 added to each of two wells. Plates were sealed with a plastic cover and incubated at 4, 10, 15, 20, and 24° C. At 72 h intervals, for 12 days, 0.1 ml of the culture fluid was removed from each well and frozen at -70° C until all aliquots were collected. At the end of 12 days, titers of all the samples were determined by TCID₅₀ assay.

SDS Polyacrylamide Gel Electrophoresis

To detect molecular weight differences in the structural proteins of the variants RB-1 and 193-110-4, partially-purified virus and high and low molecular weight markers (Bio-Rad Laboratories) were separated using SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). A 12% separating gel was prepared by combining 10.1 ml distilled water, 7.5 ml 1.5 M Tris-HCl, pH 8.8, 0.3 ml 10% (W/V) SDS, 12.0 ml 30% acrylamide (acrylamide:BIS, 30:0.8), and 0.2 ml fresh (W/V) ammonium persulfate. The mixture was degassed under vacuum for 15 min and 0.015 ml TEMED added to initiate polymerization. The mixture was poured into the electrophoresis unit (Bio-Rad Laboratories) fitted with 1.5 mm spacers and allowed to polymerize for 1.5 h. A 3% stacking gel was prepared by combining 6.3 ml distilled water, 2.5 ml 0.5 M Tris-HCl, pH 6.8, 0.1 ml 10% (W/V) SDS, 1.0 ml 30% acrylamide

(acrylamide:BIS, 30:0.8), and 0.1 ml fresh 10% (W/V) ammonium persulfate. The mixture was degassed and 0.01 ml TEMED added. A well-forming template was placed into the stacking gel during polymerization.

Concentrated virus preparations were diluted 1:2 in sample buffer (4.7 ml distilled water; 1.0 ml 0.5 M Tris-HCl, pH 6.8; 1.0 ml glycerol; 1.0 ml 10% (W/V) SDS; 0.1 ml 2-mercaptoethanol; 0.2 ml 0.05% (W/V) bromphenol blue) and heated in a boiling water bath for 5 min. A micropipettor (Gilson) was used to load 25 μ l of virus sample or 10 μ l of protein standard into wells of the stacking gel. The electrode buffer (pH 8.3) was made by combining 6.0 g Tris-base, 28.8 g glycine, and 1.0 g SDS in a flask and adding distilled water to 1 L. Electrophoresis was performed at 4 ma/gel for 16 h until the bromphenol blue tracking dye migrated to within 1 cm of the bottom of the gel.

To fix the separated proteins, gels were placed in a solution of 40% methanol/10% acetic acid for 30 min. This fixative was discarded and two changes of 10% ethanol/5% acetic acid added for 15 min each. The gel was then soaked 5 min in oxidizer (0.1% potassium dichromate, 0.015% nitric acid), followed by four rinses with distilled water. To stain the gel, silver reagent (0.2% silver nitrate) was added for 20 min. The gel was developed by successive changes (30 sec, 5 min, 5 min) of

0.28 M sodium carbonate, 0.05% formalin and the reaction stopped by transferring the gel to distilled water. Gels to be used for Western blot analysis were not stained until after electroblotting.

Western Blot Analysis

The protein specificity of monoclonal antibodies directed against IHNV antigens was analyzed by Western blots. Following electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris-base, 192 mM glycine, and 20% methanol) for 30 min. Separated proteins were electrophoretically transferred from the gels to a nitrocellulose membrane using electroblotting methods described by Towbin et al. (1979). The transfer unit (Bio-Rad Laboratories) was assembled and used according to manufacturer specifications with the following modifications. A plastic cooling coil attached to a refrigerated circulating bath (Forma Scientific) was placed in the middle slot of the transfer reservoir. A stir bar was used to assure homogeneous pH and improve heat dissipation while electrophoretic transfer was performed at 70 V for 6 h. The blotted polyacrylamide gel was silver stained to determine the efficiency of protein elution from the gel matrix. The remaining protein sites on the nitrocellulose were blocked with 5% skim milk, 0.05% sodium azide (Johnson et al., 1984) at room temperature on a rocking platform. The blocked membrane

was briefly rinsed with distilled water to remove any skim milk debris. Subsequent reactions were performed at room temperature using polyethylene bags to conserve antibody. Following blocking, the nitrocellulose membrane was incubated with 4 ml undiluted hybridoma fluid for 4-16 h, followed by three washes with shaking in 50 mM Tris-base, 150 mM NaCl, pH 7.4. To fully reduce background staining, washing was completed over a 60 min period. Goat anti-mouse IgG (gamma specific, Hyclone laboratories Inc.) or IgM (mu specific, Sigma) conjugated with horseradish peroxidase, diluted 1:500 and 1:1000 respectively, was incubated with the membrane for 1 h. The nitrocellulose was washed and incubated in the dark for 10-30 min with fresh substrate reagent. The substrate solution was prepared by combining 3 mg/ml 4-chloro-1-naphthol (Sigma) in methanol with PBS in a ratio of 1:5 and adding of H₂O₂ to 0.01%. Bands of a bluish color appeared on the nitrocellulose corresponding to the location of antigen. The membrane was washed with distilled water to remove excess substrate solution, air dried, and stored in the dark to prevent discoloration of antigen bands.

Deglycosylation of Infectious Hematopoietic Necrosis Virus

Monoclonal antibody specificity for carbohydrate moieties on viral glycoproteins was determined by Western blot analysis of deglycosylated IHNV. An equal volume of partially-purified SRCV was boiled with 0.01 ml of 1.6%

SDS for 3 min, and then cooled to room temperature. To this mixture, 0.0004 ml of 0.6 M NaH_2PO_4 - NaHPO_4 , pH 6.5, 0.0140 ml distilled water, and 1 mUnit Endoglycosidase H (Endo- β -acetylglucosaminidase H, Miles Scientific) was added. The virus was digested for 12 h at 37° C, boiled for 5 min, and an additional 1 mUnit of enzyme added. After 6 h incubation, both treated and untreated control preparations of SRCV were analyzed using 12% PAGE and Western blot analysis.

Immunofluorescence

An indirect fluorescent antibody stain was used to estimate the affinity of monoclonal antibody for wild-type and variant RB-1 and 193-110-4 antigens in infected CHSE-214 and EPC cells. Cells were grown on 18 mm coverslips for 1-3 days in 100 x 15 mm Petri dishes containing 10 ml MEM-10. The cells were infected with 10,000 TCID₅₀ of wild-type or variant virus diluted in MEM-5. Uninfected control cells received MEM-5 only. Infections proceeded 20-24 h. Cells were washed three times with PBS and fixed in cold (-20° C) 100% acetone for 10 min. Undiluted hybridoma fluid (100 μ l) was added to each coverslip and then incubated 30 min at room temperature. Unreacted immunoglobulin was removed by three changes of PBS and the coverslips reacted with 1:100 (PBS) goat anti-mouse IgG (whole molecule) conjugated with fluorescein (Hyclone Laboratories Inc.). The coverslips were rinsed three

times with PBS, mounted on microscope slides with 80% buffered glycerol, pH 8.0, and examined with a Zeiss fluorescence microscope (Carl Zeiss Inc.). For detecting internal viral antigens, cells were permeabilized with 0.5% Triton X-100 in PBS for 2 min at room temperature following acetone fixation and then stained for fluorescent microscopy.

In Vivo Studies

Care of Experimental Animals and Equipment

Rainbow trout (Salmo gairdneri) fry were used for in vivo experiments throughout this study. Three different stocks were used (Table 1).

Table 1. Sources of rainbow trout (Salmo gairdneri) used for in vivo studies.

Fish Stock	Source	Size (gm)
Tasmanian	Rainbow Trout Gardens Corvallis, OR	0.5
Oak Springs	Oak Springs Hatchery Maupin, OR	0.4
Roaring River	Roaring River Hatchery Scio, OR	0.3

Experimental animals were fed Oregon Moist Pellets ad libitum once daily and held at 12° C on dechlorinated city water at the Hatfield Marine Science Center, Newport,

Oregon. Dead fish were collected once daily, recorded, and processed for viral examination. No attempts were made to recover viral agents from animals infected with both wild-type and variant viruses.

Experiments were conducted in 80 L tanks (batch vaccinations) or 5 L plastic aquaria (small scale vaccinations and challenges). Small aquaria were each equipped with affluent and effluent tubes for constant water flow throughout experiments. Contaminated tanks were boiled for 1 h at 121^o C at the conclusion of each experiment.

Confirmation of Viral Infection

All fish were observed for typical signs induced by IHNV, including exophthalmia, petechial hemorrhaging, fecal casts, abdominal distension, lethargy, violent swimming activity, equilibrium imbalances, and a generalized darkening of the body. Dead fish from vaccination experiments were not examined for virus because these fish, in most cases, were exposed to both variant and wild-type viruses. However, fish used for virulence testing were assayed for virus.

Daily, dead fish from each tank were pooled and homogenized in HBSS at a 1:10 dilution (W/V) using a Stomacher (Dynatech Laboratories Inc.). The homogenate was centrifuged at 850 x g at room temperature for 15 min and 0.2 ml of supernatant was added to 1.8 ml of

antibiotic incubation mix containing 1000 U-1000 µg/ml penicillin-streptomycin, 500 IU/ml nystatin, and 250 IU/ml gentamicin in MEM-0. The sample was briefly mixed before incubation at 4° C for 2-4 h. Using a Pasteur pipet, one drop of mixture was added to seven wells of a 96-well microplate containing monolayers of EPC cells. Antibiotic incubation mix was added to twelve wells as a control. Plates were sealed with a plastic cover and incubated at 18° C for several days until characteristic CPE was apparent.

Comparison of Wild-type and Variant Virulence for Rainbow Trout

Waterborne vaccinations and challenges were used to mimic natural infection in fish. For initial screening of neutralization-resistant variants, replicate groups of twenty Tasmanian rainbow trout were challenged in 1 L of water with 1×10^5 TCID₅₀/ml of the variant strains or to serial dilutions of the parental wild-type strains. Exposures were for 24 h and control groups received equal volumes of MEM-5. At the end of exposure periods, water flow was resumed through aquaria. Following challenge, fish were observed for 14 days and the LD₅₀'s for RB and 193-110 determined by the method of Reed and Muench (1938). In addition, the mean day to death (MDD) for each group was calculated using the formula

$$\text{MDD} = \frac{\sum(\text{number of deaths})(\text{day of death})}{\text{total number of dead fish}}$$

In order to detect reversion of variants to wild phenotype, virus isolated from dead animals was tested by neutralization using MoAb. Variants exhibiting significantly reduced virulence for susceptible rainbow trout fry were selected for further study both in vivo and in vitro. Fifty percent lethal dose determinations were determined for RB-1 and 193-110-4 using Tasmanian rainbow trout and for RB and RB-1 using Oak Springs and Roaring River rainbow trout.

Vaccination with RB-1 and 193-110-4

Experimental fish were vaccinated with infected culture fluid containing 1×10^3 to 1×10^5 TCID₅₀/ml RB-1 or 1×10^2 to 1×10^4 TCID₅₀/ml 193-110-4. Duplicate groups of twenty fish were vaccinated by direct addition of undiluted or diluted culture fluid to 1 L of aquaria water. In two experiments, several hundred fish were batch vaccinated in 80 L tanks in 10 L of water. Different groups of fish were treated with various concentrations and for different lengths of time. In these studies, replicate groups of twenty fish were transferred to 5 L tanks for challenge at selected times postvaccination. In addition, extra fish were vaccinated to account for variant residual virulence.

Efficacy of vaccination was tested by challenging fish at selected intervals postvaccination to variant virus. In most experiments, the vaccination period was 14

days, although periods of 1, 7, and 21 days were tested in additional experiments. Undiluted, infected tissue culture fluid, with a virus titer of 1×10^5 TCID₅₀/ml, was used to challenge fish by direct addition to 1 L of aquaria water. Nonvaccinated control groups received either MEM-5 or homologous wild-type virus. All fish were exposed to virus or MEM-5 for 24 h, observed for 21 days following challenge, and then experiments were terminated. The degree of protection conferred by variants was expressed as the relative percent protection (RPS) and the value calculated as

$$1 - \frac{(\% \text{mortality of immunized fish})}{(\% \text{mortality of controls})} \times 100 = \text{RPS}$$

RESULTS

Isotype of Anti-glycoprotein Monoclonal Antibodies

Preceding this study, RB/B5 and RB/A3 isotypes were determined to be of the IgM class by Cindy Arakawa, Oregon State University. Working with IgM molecules has some difficulties including: lack of binding to protein A for purification purposes, poor Western blot and ELISA capabilities, and cross-reactiveness with salmonid cell lines. Therefore, an attempt was made to obtain neutralizing or nonneutralizing monoclonal antibodies of the IgG class. It was hoped these reagents would assist in characterizing variants. A single neutralizing (193-110/B4) and four nonneutralizing anti-193-110 monoclonal antibodies, designated B9/B4, C9/H7, E5/F11, and E12/B9, were made. All were of the IgG1 subclass except 193-110/B4 and C9/H7 which were IgG2a molecules.

Selection of Infectious Hematopoietic Necrosis Virus Variants

Prior to this work, monoclonal antibodies which neutralize the Round Butte strain were developed at Oregon State University by Cindy Arakawa. Two of these antibodies, RB/B5 and RB/A3, neutralize not only homologous virus, but also the 193-110 strain (Cathy Lannan, personal communication). Therefore, these reagents were used to select both RB and 193-110 variants. Viral surface proteins serve as targets for virus

neutralizing antibodies and, in rhabdoviruses, antibodies must bind to the glycoprotein spikes to neutralize virions. Therefore, several variants of RB and 193-110 were selected by replicating wild-type virus stocks in the presence of an overneutralizing dose of anti-RB monoclonal antibody. The number of clonings required for each variant ranged from four to ten passages (Table 2).

The frequency of variant viruses present in wild-type virus stocks was determined by dividing the titer of virus replicated in the presence of undiluted antibody by the titer of virus without antibody. Variants selected with RB/A3 and RB/B5 occurred at a frequency of 2.2×10^{-3} per infectious unit RB and 2.5×10^{-3} per infectious unit 193-110.

Variant Virus Titers

The ability of variants to replicate to normal titers was examined by performing TCID₅₀ assays. Wild-type RB and 193-110 titers reached 1×10^7 and 1×10^8 TCID₅₀/ml, respectively. Round Butte variants ranged from 1×10^7 to $1 \times 10^{7.5}$ TCID₅₀/ml and 193-110 variants from 1×10^7 to $1 \times 10^{7.7}$ TCID₅₀/ml. Round Butte-7 was an unusual exception because titers of $1 \times 10^{8.25}$ TCID₅₀/ml were consistently obtained (Table 2).

Neutralizations

Round Butte and 193-110 variants were reacted with

Table 2. Titers of IHNV variants and the number of clonings required for each variant to be 100% nonneutralized.

Variant	No. of Clonings	Titer (TCID ₅₀ /ml)
Round Butte		
-1	9	1 x 10 ^{7.5}
-2	9	1 x 10 ^{7.5}
-3	10	1 x 10 ⁷
-4	9	1 x 10 ^{7.4}
-6	10	1 x 10 ^{7.5}
-7	4	1 x 10 ^{8.25}
-9	4	1 x 10 ^{7.4}
193-110		
-1	5	1 x 10 ^{7.6}
-3	7	1 x 10 ^{7.3}
-4	7	1 x 10 ⁷
-5	7	1 x 10 ^{7.3}
-7	5	1 x 10 ^{7.7}

neutralizing monoclonal antibody 193-110/B4 to determine if the epitope recognized by this antibody overlaps, or is distinct from, the epitope operationally defined by RB/B5 and RB/A3. Because of low antibody titer, RB and 193-110 neutralization indexes (NI) were 1.2 and 1.8, respectively. Neutralization indices were consistently 0 for 193-110 variants and between 0 and 0.8 for RB variants.

Another approach was to make a bivalent mixture of RB/A3 or RB/B5 and 193-110/B4 and observe the frequency of variants in wild-type virus seeds. Each combination was used to neutralize RB and 193-110 and the frequency of variants for both was 1×10^{-3} per infectious unit virus. If the epitopes delineated by antibodies RB/A3, RB/B5, and 193-110/B4 were altered by mutations independently from each other, then the frequency of virus variants with double-point mutations would be expected to occur at a frequency in the range of the product of the single epitope variant frequencies, i. e. 1×10^{-6} . Therefore, it would appear these antibodies delineate, at least, overlapping epitopes.

To test the ability of rabbit anti-RB polyclonal serum to recognize variants, neutralizations with this serum were conducted. Neutralization indices of 3, 2.76, 2.8, and 2 were obtained for RB, RB-1, 193-110, and 193-110-4. Variants were neutralized by immune serum,

implying wild-type antigenic sites were, at least in part, retained.

Plaque Size Comparisons

The plaque sizes of variant (RB-1 and 193-110-4) and wild-type viruses were compared to determine if mutated viruses exhibit variation from wild-type plaque size. Round Butte and RB-1 plaque sizes averaged 0.52 and 0.36 mm. Plaque sizes of 193-110 and 193-110-4 were 0.19 and 0.13 mm (Figure 1).

Effects of Selected Temperatures on Virus Replication

Replication of variant and wild-type viruses at selected temperatures was examined to detect modifications in variant replication and temperature sensitivity. The EPC cell line was used because of its ability to tolerate higher incubation temperatures (up to 35° C) than the CHSE-214 cell line. Both RB-1 and 193-110-4 were capable of replication at 10, 15, and 20° C but unable to replicate at 4 and 24° C. This corresponded well with data obtained for RB and 193-110; therefore, it was concluded neither variant was temperature-sensitive. At permissive temperatures, both variants grew slower, but at the end of 12 days reached titers comparable to those of the wild types (Figure 2).

Protein Analysis in SDS Polyacrylamide Gels

To determine if the molecular weight of variants'

Figure 1. Photomicrographs of wild-type and variant plaques. The viruses are (A) Round Butte, (B) Round Butte-1, (C) 193-110, and (D) 193-110-4.

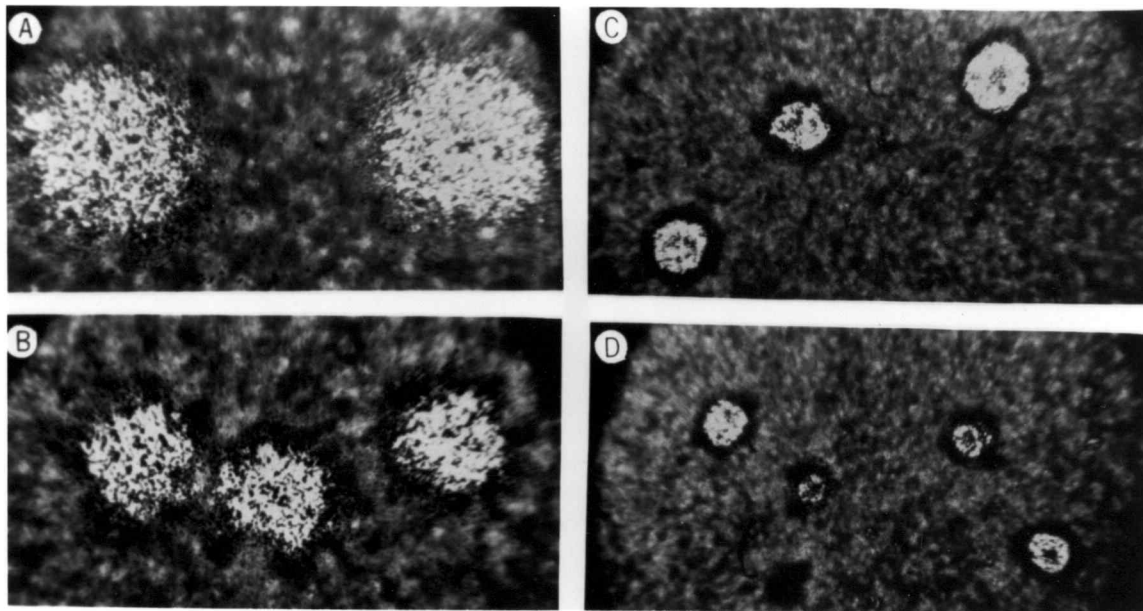


FIGURE 1

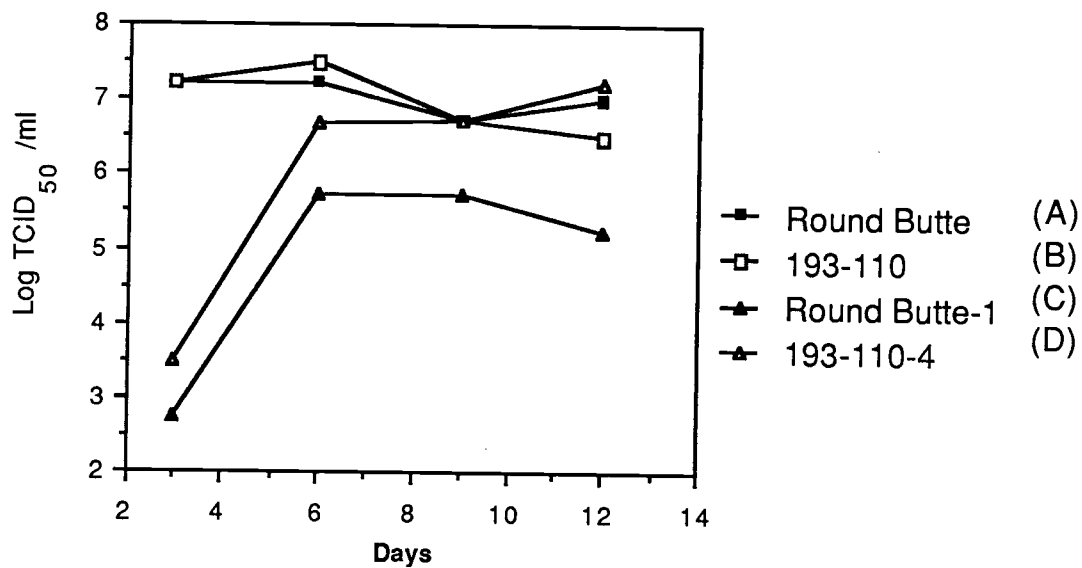


Figure 2. Concentration of infectious virus in the culture fluid of EPC cells at 15°C and infected with (A) Round Butte, (B) 193-110, (C) Round Butte-1, (D) 193-110-4.

structural proteins, especially the glycoprotein, was affected by mutation, migration of RB, RB-1, 193-110, and 193-110-4 proteins in SDS polyacrylamide gels was compared. No difference could be detected among the four strains and was supportive evidence that RB-1 and 193-110-4 retained wild-type characteristics (Figure 3).

Western Blot Analysis

Monoclonal antibodies produced against the RB and 193-110 strains of IHNV were tested for protein specificity by Western blot analysis. Antibody RB/B5 was incapable of immunologically binding to disrupted viral proteins unless concentrated and partially-purified. Monoclonal antibody RB/B5 bound to the glycoprotein molecule of the RB strain but failed to react with RB-1, 193-110, and 193-110-4. In contrast, neutralizing 193-110/B4 reacted to the glycoproteins of RB, RB-1, 193-110, and 193-110-4. Western blots utilizing RB/A3 were not attempted because of the difficulty of working with monoclonal IgM antibodies.

Nonneutralizing anti-193-110 monoclonal antibodies B9/B4, C9/H7, E5/F11, and E12/B9 were highly cross-reactive with the glycoproteins of all strains, including SRCV and H07. One of these hybridomas, B9/B4, reacted with an additional component of the viruses tested at the bromphenol blue dye front on the nitrocellulose membrane. The band was believed to represent fragments of

Figure 3. Photograph of 12% polyacrylamide gels silver stained after electrophoresis for 16 h. The samples from left to right are (A) High molecular weight markers (top to bottom: myosin, 200,000; β -galactosidase, 116,250; phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000 m. w.). (B) Round Butte. (C) Round Butte-1. (D) 193-110. (E) 193-110-4. (F) Low molecular weight markers (top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, 31,00; soybean trypsin inhibitor, 21,500; lysozyme, 14,400 m. w.).

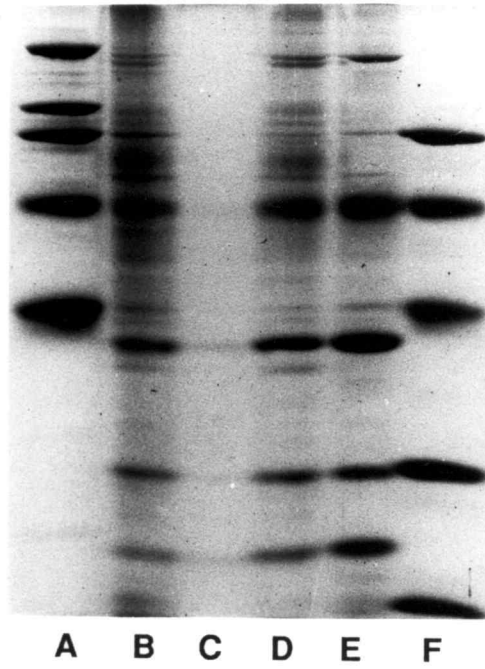


FIGURE 3

carbohydrate from the glycoproteins of these viruses. To test this hypothesis, SRCV and deglycosylated SRCV were analyzed by Western blot with monoclonal antibody B9/B4. Deglycosylated SRCV failed to react with B9/B4 and no bands formed. In contrast, nondigested virus formed bands corresponding to the glycoprotein and, presumably, carbohydrate found at the dye front on the nitrocellulose sheet.

Immunofluorescence

The binding ability of monoclonal antibodies for whole virions in infected cells was tested using an indirect fluorescent antibody technique (IFAT). The CHSE-214 cell line was used for testing monoclonal antibodies with the exception of RB/A3 and RB/B5, because these antibodies cross-reacted with these particular cells. However, noninfected EPC cells exhibited no fluorescence when reacted with the anti-RB monoclonal antibodies. Epithelioma papillosum cyprini cells infected with RB or 193-110 exhibited a positive IFAT reaction with monoclonal antibodies RB/B5 and RB/A3, as did cells infected with RB-1 or 193-110-4. Therefore, mutation(s) reduced the affinity of these antibodies for their epitopes enough to effect neutralization but not sufficiently to prevent immunofluorescent staining.

Chinook salmon embryo-214 cells infected with RB, RB-1, 193-110, or 193-110-4 reacted readily with antibodies

193-110/B4, B9/B4, C9/H7, E5/F11, and E12/B9; whereas, control noninfected cells were negative. Virus antigen was easily detected by 20 h on cell monolayers infected with either RB or 193-110. Fluorescence produced by variant antigen was weak unless cells were permeabilized with Triton X-100. This suggested that by 20 h, variant glycoprotein antigen was internal to the infected cells and indicated variants replicate slower than their homologous wild-type viruses.

Because nonneutralizing anti-193-110 monoclonal antibodies cross-reacted with several strains of IHNV, these antibodies were tested against other fish virus pathogens. Viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV) did not react with any of these monoclonal antibodies.

In Vivo Studies

Determination of the Virulence of Variant Viruses

The degree of attenuation of RB and 193-110 variants was determined in rainbow trout fry. The LD₅₀'s for Round Butte and 193-110 wild types were calculated (from the data in Table 3) using the method of Reed and Muench (1938). Because dilutions of variant and wild-type viruses did not approximate a LD₅₀ value, data points were graphed and extrapolations made to predict these values. The LD₅₀'s were estimated to be 2.25×10^3 (RB) and $4 \times$

Table 3. Virulence of Round Butte and 193-110 wild types of IHNV for Tasmanian rainbow trout fry^a held at 12^o C.

Isolate Used	Virus Concentration (TCID ₅₀ /ml)	No. Deaths/No. Tested	Percent Mortality	Mean Day to Death (MDD)
RB				
	1 x 10 ⁵	32/40	80	8.8
	1 x 10 ⁴	31/40	78	8.8
	1 x 10 ³	22/40	55	8.8
193-110				
	1 x 10 ⁵	40/40	100	8.1
	1 x 10 ⁴	38/40	95	8.6
	1 x 10 ³	34/40	85	8.4
Control ^d	---	0/40	0	---

^aMean weight of fish was 0.5 g.

^bExposure periods were 24 h in 1 L of water.

^cVirus dilutions were made in MEM-5 and added directly to aquaria water.

^dControls received MEM-5.

10^2 TCID₅₀/ml (193-110) for Tasmanian rainbow trout. To compare the virulence of wild-type with numerous variant viruses, a 1×10^5 TCID₅₀/ml dose of each variant was administered to two groups of 20 Tasmanian rainbow trout. Mortalities induced by Round Butte variants ranged from 8 to 88% and 193-110 variants from 53 to 100% (Table 4). With the exception of RB-3, 193-110-1, and 193-110-3 there was a longer mean day to death (MDD) for variants. One variant, 193-110-3, induced the same level of mortality as the homologous wild-type virus but caused a shorter MDD. Therefore, it appeared that in this one case, 193-110-3 displayed greater virulence for the host than the homologous wild-type virus. Viral variants of Round Butte and 193-110 isolated from infected fish were tested for neutralization resistance using monoclonal antibody RB/B5. All variants were nonneutralizable and reversion to wild phenotype was not detected. Variants Round Butte-1 and 193-110-4 were selected for further study, both in vivo and in vitro, because of their apparent reduction in virulence.

To determine variant LD₅₀'s, Tasmanian rainbow trout were administered 1×10^3 to 1×10^5 TCID₅₀/ml and 1×10^2 to 1×10^4 TCID₅₀/ml RB-1 and 193-110-4, respectively, for 24 h. The LD₅₀ values, estimated from Table 5, were 8.13×10^5 (RB-1) and 7.5×10^4 TCID₅₀/ml (193-110-4) which represented a 361 and 188 fold reduction in variant

Table 4. Virulence testing of Round Butte and 193-110 variants using Tasmanian rainbow trout fry^a.

Variant	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)
RB			
-1	3/40	8	10
-2	35/40	88	9.4
-3	10/40	25	6
-4	34/40	85	10.3
-6	28/40	70	9.2
-7	19/40	45	9.4
-9	20/40	50	9.4
193-110			
-1	38/40	95	7.7
-3	40/40	100	7.4
-4	21/40	53	11.3
-5	22/40	55	10.2
-7	38/40	95	8.2
Control ^d	0/40	0	---

^a Mean weight of fish was 0.5 g.

^b Titer used for variant virulence testing was 1×10^5 TCID₅₀/ml.

^c Exposure periods were 24 h in 1 L of water at 12° C.

^d Controls received MEM-5.

Table 5. Data used for estimating the LD₅₀'s of IHNV variants Round Butter-1 and 193-110-4 for Tasmanian rainbow trout fry^a.

Variant	Virus Concentration (TCID ₅₀ /ml)	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)
RB-1				
	1 x 10 ⁵	3/40	8	15
	1 x 10 ⁴	0/40	0	---
	1 x 10 ³	0/40	0	---
193-110-4				
	1 x 10 ⁴	8/40	20	11.4
	1 x 10 ³	1/40	3	11
	1 x 10 ²	1/40	3	10
Control ^c	---	0/40	0	---

^aMean weight of fish was 0.9 g.

^bExposure periods were 24 h in 1 L of water at 12° C.

^cControls received MEM-5.

virulence. Although 193-110-4 exhibited a significant reduction in virulence, the susceptibility of other rainbow trout stocks was not determined because of the residual virulence associated with this strain. The virulence of RB-1 was compared in two Oregon rainbow trout stocks, one from the Oak Springs Hatchery and another from Roaring River Fish Hatchery. Round Butte wild-type LD₅₀ values were 4.7×10^3 and 1×10^4 TCID₅₀/ml for Oak Springs and Roaring River rainbow trout. Values for RB-1 were 4.0×10^5 and 2.4×10^5 TCID₅₀/ml for these same stocks of fish (estimated from Tables 6 and 7). These results indicated an 85 and 24 fold reduction in virulence for these stocks. Round Butte-1 also induced chronic infections and caused as much as 25% scoliosis in fish of one particular group. Therefore, these two fish stocks exhibited greater susceptibility to RB-1 than Tasmanian rainbow trout.

Efficacy of Vaccination with RB-1 and 193-110-4

Vaccination experiments were conducted with Round Butte-1 and 193-110-4 to determine if variants were capable of inducing protection to wild-type virus in Tasmanian rainbow trout. Concentrations of 10,000 and 100,000 TCID₅₀/ml RB-1 protected at least 96% of the vaccinated fish. But the lower vaccinating dose of 1,000 TCID₅₀/ml resulted in only 57% protection (Table 8).

Table 6. Comparison of the virulence of Round Butte wild-type IHNV and Round Butte-1 variant for Oak Springs rainbow trout fry^a.

Virus	Virus Concentration (TCID ₅₀ /ml)	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)
RB				
	1 x 10 ⁵	37/40	93	8.1
	1 x 10 ⁴	19/40	48	9.8
	1 x 10 ³	5/40	13	9.2
RB-1				
	1 x 10 ⁵	12/40	30	12.8
	1 x 10 ⁴	4/40	10	14
	1 x 10 ³	1/40	2.5	14
Control ^c	---	0/40	0	--

^aMean weight of fish was 0.4 g.

^bExposure periods were 24 h in 1 L of water at 12° C.

^cControls received MEM-5.

Table 7. Comparison of the virulence of Round Butte wild-type IHNV and Round Butte-1 variant for Roaring River rainbow trout fry^a.

Virus	Virus Concentration (TCID ₅₀ /ml)	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)
RB				
	1 x 10 ⁵	30/40	75	7.3
	1 x 10 ⁴	27/40	68	8.9
	1 x 10 ³	20/20	50	10.3
RB-1				
	1 x 10 ⁵	7/39	18	9.3
	1 x 10 ⁴	6/38	16	9.3
	1 x 10 ³	4/39	10	8.3
Control ^c	---	0/40	0	---

^aMean weight of fish was 0.3 g.

^bExposure periods were 24 h in 1 L of water at 12° C.

^cControls received MEM-5.

Table 8. Efficacy of three different vaccination doses of IHN variant Round Butte-1 in Tasmanian rainbow trout fry^a to a 1×10^5 TCID₅₀/ml challenge of homologous wild-type IHNV.

Vaccination Dose (TCID ₅₀ /ml)	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)	Percent Protection
1×10^5	1/37	3	13	96
1×10^4	1/40	3	20	96
1×10^3	11/40	30	13	57
Control ^b	25/40	63	13	--
Control ^c	0/40	0	--	--

a
Mean weight of fish was 0.9 g.

b
Fish were vaccinated with MEM-5 and challenged with 1×10^5 TCID₅₀/ml Round Butte wild type added directly to aquaria 14 days later.

c
Fish were vaccinated and challenged with MEM-5.

d
Exposure periods were for 24 h in 1 L of water at 12° C.

Each group of fish vaccinated with 193-110-4 effectively withstood challenge, while 95% of the nonvaccinated controls died. The two lower vaccinating doses of 100 and 1,000 TCID₅₀/ml protected 100% of the fish challenged. Although, approximately 16% of the fish vaccinated with the highest dose (10,000 TCID₅₀/ml) died from viral infection during the vaccination period, 84% of fish were protected when challenged (Table 9).

To determine if the mechanism for protection was interference, experiments were performed using Oak Springs and Roaring River rainbow trout. One-hundred percent of the Oak Springs fish succumbed to viral infection when challenged one day postvaccination. Interference did not appear to be the mechanism for protection in these fish, because animals vaccinated with RB-1 at a concentration of 100,000 TCID₅₀/ml were protected when challenged on days 7, 14, and 21 (Table 10). But, the protection conferred at these periods was only 25-35% and decreased with time postvaccination. Protection at the lower vaccination doses, 1,000 and 10,000 TCID₅₀/ml, was negligible. All groups of the nonvaccinated fish experienced high mortality when exposed to Round Butte wild type. Those challenged on days 1, 7, and 14 had 100% mortality; on day 21 postvaccination, 78% of the control fish died.

Roaring River fish vaccinated with RB-1 at concentrations of 10,000 and 100,000 TCID₅₀/ml exhibited

Table 9. Efficacy of three different vaccination doses of IHN_V variant 193-110-4 in Tasmanian rainbow trout fry^a to a 1×10^5 TCID₅₀/ml challenge of homologous wild-type IHN_V.

Vaccination Dose (TCID ₅₀ /ml)	No.Deaths/No.Tested	Percent Mortality	Mean Day to Death (MDD)	Percent Protection
1×10^4	5/32	16	12.6	84
1×10^3	0/39	0	---	100
1×10^2	0/39	0	---	100
Control ^b	38/40	95	10	---
Control ^c	0/40	0	---	---

a

Mean weight of fish was 0.9 g.

b

Fish were vaccinated with MEM-5 and challenged with 1×10^5 TCID₅₀/ml 193-110 wild type added directly to aquaria 14 days later.

c

Fish were vaccinated and challenged with MEM-5.

d

Exposure periods were for 24 h in 1 L of water at 12° C.

Table 10. Efficacy of selected concentrations of IHNV variant RB-1 to prevent IHN in Oak Springs rainbow trout^a at different periods post-vaccination.

Vaccination Dose (TCID ₅₀ /ml)	Days Post-vaccination Prior to Challenge	No. Deaths No. Tested	Percent Mortality	Percent Protection
1 x 10 ⁵	1	40/40	100	0
	7	26/40	65	35
	14	28/40	70	30
	21	7/12	58	25
1 x 10 ⁴	1	40/40	100	0
	7	33/40	83	18
	14	37/40	93	8
	21	22/33	67	14
1 x 10 ³	1	40/40	100	0
	7	40/40	100	0
	14	35/40	88	13
	21	35/40	88	0
Control ^b	1	40/40	100	--
	7	40/40	100	--
	14	40/40	100	--
	21	31/40	78	--

a Mean weight of fish was 0.4 g

b Controls were challenged with 1 x 10⁵ TCID₅₀/ml Round Butte wild type.

c Three-hundred fish were vaccinated in 10 L of water at 12° C for 24 h.

d Vaccinated fish were challenged with 1 x 10⁵ TCID₅₀/ml Round Butte wild type in 1 L of water for 24 h.

Table 11. Efficacy of selected concentrations of IHN variant RB-1 to prevent IHN in Roaring River rainbow trout^a at different periods post-vaccination.

Vaccination Dose (TCID ₅₀ /ml)	Days Post-vaccination Prior to Challenge	No. Deaths No. Tested	Percent Mortality	Percent Protection
1 x 10 ⁵	1	27/40	68	18
	7	15/40	38	48
	14	8/40	20	58
	21	1/18	6	78
1 x 10 ⁴	1	40/40	100	0
	7	17/40	43	41
	14	11/40	28	42
	21	4/40	10	60
1 x 10 ³	1	33/40	83	0
	7	18/40	45	40
	14	22/40	55	0
	21	8/40	20	20
Control ^b	1	33/40	83	--
	7	29/40	73	--
	14	19/40	48	--
	21	10/40	25	--

a Mean weight of fish was 0.3 g.

b Controls were challenged with 1 x 10⁵ TCID₅₀/ml Round Butte wild type.

c Three-hundred fish were vaccinated in 10 L of water at 12° C for 24 h.

d Vaccinated fish were challenged with 1 x 10⁵ TCID₅₀/ml Round Butte wild type in 1 L of water for 24 h.

Table 12. Selected RB-1 vaccination periods to protect Oak Springs rainbow trout^a to a 1×10^5 TCID₅₀/ml challenge of homologous wild-type virus.

Vaccination Dose (TCID ₅₀ /ml)	Vaccination Period (Hours)	No. Deaths No. Tested	Percent Mortality	Percent Protection
1×10^5	1	16/32	50	50
	12	15/27	56	44
	24	28/40	70	30
1×10^4	1	28/36	78	22
	12	19/29	66	34
	24	37/40	93	8
1×10^3	1	23/39	59	41
	12	28/39	72	28
	24	35/40	88	13
Control ^b	--	40/40	100	--

a Mean weight of fish was 0.4 g.

b Controls were challenged with 1×10^5 TCID₅₀/ml Round Butte wild type after 14 days.

c Fish were vaccinated in 10 L of water at 12° C for 24 h and removed at 1, 12, and 24 h.

d Vaccinated fish were challenged with 1×10^5 TCID₅₀/ml Round Butte wild type in 1 L of water for 24 h.

Table 13. Selected RB-1 vaccination periods to protect Roaring River rainbow trout^a to a 1×10^5 TCID₅₀/ml challenge of homologous wild-type virus.

Vaccination Dose (TCID ₅₀ /ml)	Vaccination Period (Hours)	No. Deaths No. Tested	Percent Mortality	Percent Protection
1×10^5	1	9/33	28	43
	12	5/26	19	60
	24	8/40	20	58
1×10^4	1	13/27	35	26
	12	6/35	17	64
	24	11/40	28	42
1×10^3	1	13/39	33	31
	12	9/37	24	49
	24	22/40	55	0
Control ^b	--	19/40	48	--

^a Mean weight of fish was 0.3 g.

^b Controls were challenged with 1×10^5 TCID₅₀/ml Round Butte wild type after 14 days.

^c Fish were vaccinated in 10 L of water at 12° C for 24 h and removed at 1, 12, and 24 h.

^d Vaccinated fish were challenged with 1×10^5 TCID₅₀/ml Round Butte wild type in 1 L of water for 24 h.

some degree of protection on days 7, 14, and 21 (Table 11). Unlike the experiments with Oak Springs fish, protection increased with time postvaccination before challenge. Similar results might have been obtained for Oak Springs fish if those animals had not been challenged with excessive wild-type doses. No protection was afforded to animals challenged on day 1; therefore, interference was not the mechanism for protection. Nonvaccinated fish exposed to Round Butte wild type demonstrated mortalities of 83% (day 1), 73% (day 7), 48% (day 14), and 25% (day 21). Therefore, fish susceptibility decreased rapidly with time.

Studies were undertaken using Oak Springs and Roaring River rainbow trout to determine if efficacy of vaccination was affected by vaccination time to different concentrations of RB-1. Both fish stocks were vaccinated for 1, 12, and 24 h and each group challenged 14 days postvaccination. Results were equivocal, but there were indications that protection was not affected by (Tables 12 and 13) the time fish were immersed in the vaccine preparation nor by the concentration of variant virus.

DISCUSSION

Several Round Butte and 193-110 variants were selected from wild-type virus stocks by their inability to be neutralized by glycoprotein-specific monoclonal antibody, RB/B5. To determine if variants had altered glycoproteins which affected viral virulence and/or phenotype, several characteristics of two of these variants, RB-1 and 193-110-4, were studied in vitro and in vivo. These studies suggested that virulence of IHNV was associated, in part, with the portion of the glycoprotein recognized by anti-Round Butte monoclonal antibody RB/B5.

Only glycoprotein-specific antibodies have neutralizing activity for rhabdoviruses. However, to confirm the protein specificity of neutralizing and nonneutralizing IHNV monoclonal antibodies used in this study, Western blot analysis was performed. All monoclonal antibodies reacted with the glycoproteins of variant and wild-type virus except antibody RB/B5 which reacted only with the RB strain. Reduced avidity of the RB/B5 antibody for RB-1, 193-110, and 193-110-4 glycoproteins may have allowed loss of antibody during washing steps. In addition, nonneutralizing antibodies reacted immunologically with the glycoproteins of SRCV and H07, indicating that highly conserved areas exist on IHNV glycoproteins. Lefrancois (1984) reported the ability of several nonneutralizing anti-VSV monoclonal antibodies to

react with both the Indiana and New Jersey serotypes which could be easily distinguished by neutralization. In that study, by ELISA, nonneutralizing monoclonal antibodies had lower affinity than neutralizing antibodies for homologous intact virions. The author suggested this effect may be a result of differences between exposed neutralization determinants and the more highly conserved, inaccessible epitopes recognized by the nonneutralizing antibodies. In my study, one nonneutralizing monoclonal antibody, B9/B4, reacted with glycoprotein carbohydrate moieties in Western blots. Therefore, most of the monoclonal antibodies specific for IHNV appeared to be directed against the protein portions of the glycoprotein molecule because only one antibody was observed to react with carbohydrate.

Variants of RNA viruses have been readily generated *in vitro* by other workers because of the high mutation rate of single-stranded RNA genomes (Holland et al., 1982). Influenza, rabies, picornavirus, and paramyxovirus variants have been selected at frequencies between 10^{-4} to 10^{-5} (Portner et al., 1980; Wiktor and Koprowski, 1980; Emini et al., 1983; Sherry and Rueckert, 1985; Yewdell and Gerhard, 1982). In several studies by other investigators, selection of variants was performed using a single-step selection procedure which incorporated high-titer monoclonal antibody (Gerhard and Webster, 1978; Lafon et al., 1983; Wiktor and Koprowski, 1980). In my

study, wild-type IHNV present during the initial clonings was not fully neutralized because of the relatively low titer of the monoclonal antibody. Because multiple clonings were required to fully inactivate and remove any wild-type virus, it was not possible to accurately estimate the frequency of IHNV variants in the wild stocks.

Studies comparing the characteristics of RB-1 and 193-110-4 to homologous wild-type virus in vitro were conducted. The titers of the variant strains were normal; differences among the structural proteins were not detected; and variants were not temperature-sensitive mutants. Variants were also neutralized by hyperimmune serum, a characteristic of viruses which retain wild-type antigenic determinants. Variation in the carbohydrate moieties of variant and wild-type glycoproteins could not be detected with nonneutralizing monoclonal antibody, B9/B4. These observations indicated that RB-1 and 193-110-4 retained several wild-type characteristics that were not adversely affected by mutation.

Although variants had many similarities to wild-type virus, some differences were detected. Plaques formed by RB-1 and 193-110-4 variants were approximately 69% the size of wild-type plaques. Reduction in viral plaque size has been attributed to reduced replication rate (Simizu et al., 1973; Strauss and Strauss, 1980) acidic overlay (Vogt

et al., 1957), age of cells in assay (Burke and Mulcahy, 1980), and electrostatic interaction of charged virions with sulfated polysaccharides in agar overlay (Liebhaber and Takemoto, 1963). The smaller plaques formed by the IHNV variants were probably not affected by overlay conditions because methylcellulose is not acidic nor does it contain sulfated polysaccharides. Wagner et al. (1963) reported small plaque mutants of the VSV Indiana and New Jersey serotypes showed longer times for viral production than wild-type virus. Similarly, the size of plaques formed by wild-type and variant strains of IHNV probably reflected their relative rates of replication because variants RB-1 and 193-110-4 replicated more slowly than the wild-type viruses at permissive temperatures. Immunofluorescent antibody studies supported these data because at 20-24 h postinfection, glycoproteins of variants were observed intracellularly; whereas, wild-type glycoprotein had become extracellular. Slower rates of variant replication would explain why wild-type strains predominate in cell culture when no selective pressure is applied.

Variants resistant to neutralization serve as markers for individual epitopes and based on their differential reactivity with monoclonal antibodies, an epitope map can be constructed. Because only two neutralizing monoclonal antibodies were available for this study, the maximum

number of antigenic determinants that could have been delineated was two. However, monoclonal antibody, 193-110/B4, failed to neutralize both the RB and 193-110 variants. These findings suggested that the neutralization epitopes recognized by monoclonal antibodies RB/B5 and 193-110/B4 overlap and may be identical. However, monoclonal antibody competition assays should be conducted to definitively prove the two epitopes overlap. Neutralization indices were between 0-0.8 and values less than one are not considered to be significant (Rovozzo and Burke, 1973). Further evidence that monoclonal antibodies RB/B5 and 193-110/B4 were directed against the same or overlapping epitopes was demonstrated when variants were selected at single and not double-point mutational rates in the presence of both neutralizing antibodies. These data suggested that the wild-type IHNV strains, RB and 193-110, share a common neutralization determinant on their glycoprotein molecules. Because monoclonal antibodies have only been developed against this single epitope, it may be that IHNV expresses a single immunodominant determinant on the glycoprotein.

Although the glycoprotein of variant viruses was not neutralized or did not react in Western analyses by RB/B5, the binding affinity of this antibody was examined. The glycoproteins in variant infected cells immunologically

reacted with antibody RB/B5. Holland et al. (1983) reported that some neutralization-resistant herpes simplex virus type 1 variants retain their ability to be immunoprecipitated by neutralizing monoclonal antibody. These results showed that structural changes in a neutralization epitope can convert it into an epitope that is capable of binding a neutralizing antibody without neutralization. Therefore, the actual changes at this site within the variant glycoprotein were probably very minor because the antibody still recognized important epitope sequences which allowed binding to occur.

In addition to in vitro studies of RB-1 and 193-110-4, virulence of several variants was tested in vivo. The mortalities induced by the battery of variants tested was variable and at least one appeared to have greater virulence for the host than the homologous wild-type virus. This suggested that not all amino acid changes in the epitope conferring neutralization-resistance lead to an avirulent phenotype. Molecular studies of these virulent variants may reveal information about how new virulent strains of IHNV evolve in the environment.

From the battery of variants, two were chosen for more in depth studies in vivo. The 193-110-4 strain was not as attenuated for rainbow trout as RB-1; therefore, other areas within the epitope or at a separate epitope may be critical for determining 193-110 virulence. RB-1

exhibited variable virulence for Tasmanian, Oak Springs, and Roaring River rainbow trout stocks. Virus isolated from fish in these experiments was still neutralization-resistant; therefore, reversion of RB-1 to wild phenotype did not explain differences seen among stocks of fish. Differences in susceptibility among stocks of chinook salmon (Chen, 1983; Wertheimer and Winton, 1982) and within the same stock of sockeye salmon (Oncorhynchus nerka) (Amend and Nelson, 1977) have been reported. Subtle genetic differences in the viral receptors of these test animals may explain variation in their susceptibilities to IHNV. In two of the trout stocks, Oak Springs and Roaring River, infections were chronic and a number of fish exhibited spinal deformities. Wild-type infected fish did not exhibit scoliosis probably because infections were acute and killed animals before deformities could be induced. This high prevalence of scoliosis may reflect altered tissue tropism of the variant virus.

Vaccination experiments were conducted to determine if RB-1 and 193-110-4 could induce protection against challenge by wild-type virus in rainbow trout. At higher vaccine doses, variant RB-1 protected at least 96% of the fish against wild-type challenge. A low dosage (1×10^3 TCID₅₀/ml) afforded less protection perhaps because there was insufficient viral replication in the host to induce a

response. Variant 193-110-4 induced good protection at low vaccination dosage, probably because residual virulence allowed some limited replication to occur.

Experiments were conducted to determine if the protection induced by RB-1 was a result of a specific immune response or blockage of primary target sites in the host. Neither Oak Springs nor Roaring River rainbow trout were protected when challenged one day postvaccination. Viral interference did not appear to be the protective mechanism in these fish because superinfection was not prevented. In contrast to these findings, Bernard et al. (1983) demonstrated that an attenuated, thermoresistant variant (F25-21) of VHSV protected fish as early as 24 h after vaccination against challenge by the wild-type virus strain. They did not determine if this response was a result of interferon production or interference. Because rainbow trout in my study were afforded protection when challenged 7, 14, and 21 days postvaccination, stimulation of an IHNV-specific immune response was likely. Assays for anti-IHNV antibody in the serum of vaccinated fish were not performed because of the difficulty of obtaining sufficient quantities of fish serum for viral neutralizations.

Vaccination periods to different concentrations of RB-1 were tested using Oak Springs and Roaring River rainbow trout. Data were equivocal; but, there were

indications that protection was not affected by either vaccine concentration or period. Tebbitt (1976) found that vaccination periods of 12, 24, and 48 h were effective in protecting kokanee and chinook salmon when vaccinated with an attenuated waterborne IHNV vaccine. But, higher vaccine doses were required for the shorter vaccination periods to achieve the same level of protection as fish exposed for a 48 h period.

One of the most important areas of research regarding IHNV variants will be to compare the amino acid sequence of the glycoprotein of variant and wild-type strains of IHNV. In this study, viral virulence was demonstrated to be linked to a site of the IHNV glycoprotein recognized by monoclonal antibody RB/B5. By locating amino acid changes in the peptide sequence of both virulent and avirulent variants, areas critical for determining virulence can be delineated. Once characterized, site-directed mutagenesis may be utilized to delete or remove amino acids critical for IHNV virulence. Meanwhile, variants should be further attenuated using different monoclonal antibodies or by serially passing the viruses in cell culture. Once further attenuated, virulence of variants would then be retested in vivo and if avirulent, used in vaccination trials. These studies would require testing of parameters such as vaccine dose, vaccination period, species susceptibility, and cross-protection

against other strains of IHNV or disease agents. In conjunction with protection studies, the parameters of the immune response should be examined.

Other areas of research involve additional characterization of variant viruses. Histological studies of variant-infected fish may demonstrate the role of particular amino acids in tissue tropism and ultimately in pathogenesis. Information regarding the viral receptor binding sites on both virions and target sites in the host may be useful in determining why the disease is restricted primarily to young fish and to particular species and stocks of salmonids. In addition, epitope analysis with a greater number of monoclonal antibodies to the strains used in this study and selection of variants against other IHNV strains will determine if modifications to other regions of the glycoprotein are associated with loss of virulence.

SUMMARY AND CONCLUSIONS

1. Several Round Butte and 193-110 variants were selected *in vitro* using glycoprotein-specific monoclonal antibodies. Four to ten clonings in the presence of antibody were required for variants to be 100% nonneutralized.
2. Variant titers were normal and neutralization by immune rabbit serum was efficient.
3. Monoclonal antibody 193-110/B4, which neutralizes the the Round Butte and 193-110 wild types, failed to neutralize Round Butte and 193-110 variants. Therefore, the epitope recognized by 193-110/B4 overlaps the epitope neutralized by the selecting antibody, RB/B5.
4. The plaques of variant viruses RB-1 and 193-110-4 were approximately 31% smaller than the plaques of the homologous wild-type virus.
5. Replication of RB-1 and 193-110-4 at 10, 15, and 20° C was slower than the parent viruses. All viruses replicated poorly at 4 and 24° C; therefore, variants were probably not temperature-sensitive mutants.
6. No differences could be detected among the structural proteins of RB, RB-1, 193-110, and 193-110-4 on SDS

polyacrylamide gels.

7. In Western blots, RB-1, 193-110, and 193-110-4 failed to immunologically react with monoclonal antibody RB/B5, but this antibody did bind to the glycoprotein of RB. In contrast, monoclonal antibody 193-110/B4 reacted with the glycoprotein of all the tested viruses as did several nonneutralizing anti-193-110 monoclonal antibodies. In addition, nonneutralizing antibodies bound to the glycoproteins of SRCV and H07.
8. Anti-Round Butte and anti-193-110 monoclonal antibodies reacted with RB-1 and 193-110/B4 infected fish cells. Therefore, altered antibody affinity prevented neutralization but not immunofluorescent staining. Variant glycoprotein was not readily detected on the surface of infected cells at 20-24 h postinfection but was found internally in permeabilized cells. This was a further indication that variants replicate at a slower rate.
9. Variants RB-1 and 193-110-4 demonstrated reduced virulence for Tasmanian rainbow trout. RB-1 also exhibited a reduction in virulence for Oak Springs and Roaring River rainbow trout. There was variation in stock susceptibility to RB-1 but Tasmanian rainbow trout were the most resistant.

10. Vaccinations with RB-1 and 193-110-4 afforded high levels of protection against challenge by homologous wild-type virus in susceptible Tasmanian rainbow trout fry. However, protection was considerably less for Oak Springs and Roaring River rainbow trout. For the latter two fish stocks, interference did not appear to be the mechanism for protection.

11. IHNV virulence was associated, in part, with the region of the glycoprotein recognized by monoclonal antibody RB/B5. Consequently, these variants could be used to further study viral mechanisms of virulence, pathogenesis, and antigenicity.

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