AN ABSTRACT OF THE THESIS OF

Kyoung Chul Park for the degree of Doctor of Philosophy in Microbiology presented on December 12, 2000. Title: CHARACTERIZATION OF AN INHIBITOR ("6S") OF INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV) IN NORMAL RAINBOW TROUT SERUM (RTS) AND ITS EFFECTS ON THE VIRUS

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Paul W. Reno

The characteristics of an inhibitor of infectious pancreatic necrosis virus (IPNV) found in normal rainbow trout serum (RTS) were studied. The serum inhibitor had a molecular weight of approximately 150 kDa and was dependent on divalent cations, either Ca^{2+} or Mg^{2+}. It was stable at temperatures up to 50°C and at a pH range between 4–10. The inhibitor directly inactivated the virus and the inhibition level was dependent on cell densities and on the time at which virus was exposed to RTS. The level of virus inhibition by RTS was altered by the cell line in which virus was produced. IPNV was more efficiently inhibited by RTS in salmonid cell lines than in non-salmonid cell lines. Most of the salmonid sera tested showed inhibition, while non-salmonid sera did not inhibit IPNV replication. Rainbow trout continuously showed a significant level of inhibition in their serum after 23 weeks post hatch.

Three isolates of IPNV were passaged five times in RTG-2 cells with either MEM-10 or MEM-10 with 1% rainbow trout serum and virus from each passage were tested for RTS sensitivity *in vitro* and virulence *in vivo*. The mortality level in brook trout

fry was highly variable during viral passages, ranging between 30-89%. The RTS sensitivity and virulence were changed during viral passages, and these changes were dependent on cell culture conditions and IPNV isolate used. It was found that an IPNV crayfish isolate passaged in RTG-2 cells with MEM-10 showed significantly increased RTS sensitivity. This was, however, not correlated with decreased virulence. All three isolates showed identical antigenicity patterns with a panel of 11 monoclonal antibodies, irrespective of viral passage conditions.

Clones prepared from an IPNV-Jasper (Ja) population which had been twice passed through brook trout were heterogeneous with respect to RTS sensitivity, serotype, and cDNA sequences. Eight percent of clones (4/50) were very sensitive to RTS (Ja-S), as was the parent strain, and eighty four percent of clones (42/50) showed a mid-range of RTS sensitivity. The final eight percent of clones (4/50) were RTS resistant (Ja-R). Enzyme immunodot assay revealed that Ja-S clones and Ja-R clones differed by several epitopes. Ja-S and Ja-R had significant differences in their cDNA sequences for the capsid protein VP2. These two strains shared 80.7% and 86% identity in nucleic acid and in amino acid sequences, respectively.

CHARACTERIZATION OF AN INHIBITOR ("6S") OF INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV) IN NORMAL RAINBOW TROUT SERUM (RTS) AND ITS EFFECTS ON THE VIRUS

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CONTRIBUTION OF AUTHORS

The research presented in this thesis was performed under the direction of Dr. Paul Reno in his laboratory at the Coastal Oregon Marine Experimental Station, Hatfield Marine Science Center, Department of Microbiology, Oregon State University, Newport, Oregon. Dr. Paul Reno was also involved in the design, analysis, and writing of each manuscript.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

Page

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

Figure Pages

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

Figure Pages

LIST OF FIGURES (CONTINUED)

Figure Pages

LIST OF TABLES

LIST OF TABLES (CONTINUED)

DEDICATION

I dedicate this thesis to my Mom, Dad, and family in Korea and to my wife Kyung-Sook.

Their love and support always encouraged me to explore.

CHARACTERIZATION OF AN INHIBITOR ("6S") OF INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV) IN NORMAL RAINBOW TROUT SERUM (RTS) AND ITS EFFECTS ON THE VIRUS

CHAPTER 1

THESIS INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is an unenveloped, bisegmented, and double-stranded RNA virus (Dobos 1976). The virus is a member of the family Birnaviridae and can cause mortality as high as 90-100%, in 1-4 month-old rainbow trout *Oncorhynchus mykiss* (Frantsi & Savan 1971, McAllister 1983). The IPNV-like diseases have been expanding worldwide since the first report from the Maritime Provinces, Canada (M'Gonigle 1941). These viruses have devastating effects on not only salmonid species but also non-salmonid species (Reno 1999). To date, aquatic birnaviruses have been detected in nearly 80 species of aquatic animals from over 24 countries (Reno 1999). Aquatic birnaviruses are grouped into serogroups A and B in which serogroup A containing more than 200 isolates is composed of nine serotypes (A_1 to A_9), while serogroup B has only one serotype (B_1) having fewer than 10 isolates (Hill and Way 1995).

It has been reported that an anti-IPNV inhibitor, called "6S inhibitor", is present in rainbow trout serum (RTS) unexposed to IPNV (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974). The serum inhibitor had a sedimentation coefficient of approximately 6S by ultracentrifugation and, thus, is smaller than fish antibody (Dorson & de Kinkelin 1974). Kelly and Nielsen (1985) found that viral adsorption to cells was

inhibited to some extent in the presence of RTS and assumed that this inhibition is not due to induction of interferon or binding to cellular receptors. Not all virus isolates tested are inhibited by RTS and the inhibition is not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Okamoto et al. 1983a, Ögüt 1995). It has been suggested by some researchers that the ability of IPNV strains to replicate in the presence of normal trout serum is an important aspect of virus pathogenesis and can be considered a virulence factor (Hill & Dixon 1977, Hill 1982). It has been reported that both the 6S sensitivity and the virulence of IPNV can be changed by passaging virus *in vitro* in the presence of RTS (Hill and Dixon 1977, Hill 1982, Ögut 1995). Hill and Dixon (1977) noted that the acquisition of 6S sensitivity by IPN virus may not be a permanent mutation or an adaptation process, but a simple selection of a fast-growing tissue culture variant from a mixtured virus population, and that this process can be suppressed or even reversed by growth of the virus in the presence of normal trout serum.

Although some researchers acknowledged the importance of serum inhibition in pathogenesis of IPNV, the characteristics and mechanism of action of a 6S inhibitor are still ambiguous. The goal of the studies in this thesis was to investigate characteristics of 6S inhibitor, the inhibition mechanism, the effect of viral passages on virulence and RTS sensitivity of IPNV, and any genetic difference between RTS sensitive strain and RTS resistant strains of virus.

Chapter 2 is a review of literature which provides information on IPN disease, IPNV, and current knowledge related to "6S" inhibitor in normal rainbow trout serum against IPNV.

In chapter 3, the characteristics of an inhibitor of infectious pancreatic necrosis virus (IPNV) found in RTS, including molecular size, stability at different pH and temperatures, ontogeny in trout and effect of cations on the activity of RTS inhibitor were investigated.

In chapter 4, a series of experiments was conducted to determine the mechanism of RTS inhibitor of IPNV and the characteristics related to RTS inhibitory activity.

Chapter 5 describes the effect of *in vitro* passage of IPNV on virulence and sensitivity of the virus to RTS. In this study, the effect was determined at each of multiple viral passages. Another aim in this chapter was to determine if the change in RTS susceptibility during viral passages *in vitro* is related to epitope alteration.

In Chapter 6, we determined whether a subset of the virus population was heterogeneous in terms of RTS sensitivity and genetic sequences. First, the RTS sensitivity of clones from the IPNV-Jasper isolate was tested. Second, the epitope patterns of clones with two different sensitivities were determined. Third, the cDNA sequences of VP2 region of two strains, RTS sensitive or RTS resistant, were compared.

CHAPTER 2

LITERATURE REVIEW

INFECTIOUS PANCREATIC NECROSIS DISEASE

History

Infectious pancreatic necrosis virus (IPNV) is a member of the family Birnaviridae. The first report of IPNV-like disease was described by M'Gonigle (1941). He reported a recurrent disease occurrence in various hatcheries in maritime Canada. Whirling behavior and high mortality in brook trout *Salvelinus fontinalis* fry were observed. At that time, the symptom was described as "acute catarrhal enteritis". A similar outbreak occurred in brook trout in West Virginia. Infected trout fry showed acute pancreatic necrosis, and the disease was renamed as infectious pancreatic necrosis (Wood et al. 1955).

The infectious nature of IPN was proved by Snieszko et al. (1959). Brook trout fry with no signs of IPN showed signs of IPN when trout were exposed to tissue homogenates of infected fish. The viral nature of IPN was first confirmed, fulfilling Rivers' postulates (Rivers, 1937), by Wolf et al. (1961). Tissue extract was obtained from diseased brook trout, then the filtrate was inoculated on cells. Cytopathic effect (CPE) was observed in one day and cell-culture supernatant was serially passaged. Brook trout fry showed typical signs of IPN disease when the fish were exposed to tissue-culture supernatant. After the first isolation in North America from brook trout *Salvelinus fontinalis* (Mitchill) (Wolf et al,1960), the isolation of infectious pancreatic necrosis virus

(IPNV) was soon followed by isolation in rainbow trout *Oncorhynchus mykiss* (Walbaum), brown trout *Salmo trutta* L. and other salmonid fish (MacAllister and Owens, 1995).

Geographic and host range

The IPNV-like disease has been expanding worldwide since the first report from the Maritime Provinces, Canada (M'Gonigle 1941) and then in the eastern USA (Wood et al. 1955). All of the early reports on IPNV were limited to trout, such as brook trout *Salvelinus fontinalis* (Wood et al. 1955, Snieszko et al. 1957, 1959, Wolf et al. 1960), rainbow *Oncorhynchus mykiss* and brown trout *Salmo trutta* (Ball et al. 1971, Sano 1971). Thus, it was considered that IPNV caused devastating effects only in salmonid species. Since 1969, however, IPNV-like birnavirus has been reported from many non-salmonid species. The first report of disease caused by IPNV-like birnavirus in non-salmonids from Japan in Japanese eels *Anguilla japonica* in 1969 (Sano et al. 1981); later IPNV carrier was reported from a population of healthy white suckers *Catastomus commersoni* downstream of an IPN-positive Canadian fish hatchery (Sonstegard et al. 1972). Stephens et al. (1983) isolated IPNV-like birnavirus from Altantic menhaden *Brevoortia tyrannus* suffering from a "spinning" disease in Chesapeake Bay. In Japan, IPNV-like birnavirus causing disease was reported from yellowtail *Seriola quinqueradiata* fry and fingerlings (Sorimachi and Hara 1985) and Japanese flounder *Paralichthys olivaceus* (Kusuda et al. 1989). The virus was also isolated from snakehead

fish *Ophicephalus striatus* in Thailand, and eyespot barb *Hampala dispar* in Laos (Wattanavijarn et al. 1988). In France, an IPNV-like birnavirus was also isolated from seabass *Dicentrarchus labrax* (Bonami et al. 1983) and turbot *Scophthalmus maximus* (Castric et al 1987). IPNV-like birnavirus was also isolated from invertebrates including molluscans (Hill, 1982) and crustacea (Bovo et al. 1984). To date, aquatic birnaviruses have been detected in nearly 80 species of aquatic animals from over 24 countries (Reno 1999).

Signs of IPN disease and epidemiology

The characteristic behavioral signs of IPN disease are anorexia and whirling behavior alternating with prostration which is commonly a terminal sign (Wolf 1966). External signs of IPN disease include overall darkening, variable exopthalmia, abdomen distension, petechial hemorrhages on ventral areas, and pale gills. Internal signs of the disease include lack of any food in the gut, which contains a clear milky mucus in the stomach and anterior intestine, pale spleen, heart, kidneys and liver, and petechial hemorrhage in the pyloric caeca and adipose tissue (Wolf 1988). Histopathologically, the effects of IPN infection are marked pancreatic inflammation and necrosis of both the islet and the acinar regions of the pancreas (Wolf 1988).

IPNV can be transmitted through both vertical and horizontal mechanisms. Infected fish shed the virus in the feces and urine, and can then be transmitted mostly through ingestion or contact with the gills (Wolf 1988). Most survivors of IPN disease

become carriers shedding the virus, probably for life, in their feces and in their seminal and ovarian fluids during spawning (Hill 1982). IPNV is known to be vertically transmitted through eggs, and iodophor treatment of eggs was not successful in preventing vertical transmission of IPNV (Wolf et al. 1968, Bullock et al. 1976).

Although the time course of IPN disease is dependent on factors such as host species and age, virus strain, and water temperature, disease signs generally appear in about 3-5 days or 8-10 days after viral exposure of fry or fingerlings, respectively (Noga 1996). Although mortality from IPNV can occur over a period of 4-6 weeks after initial exposure to the virus, peak mortality usually occurs from 7 to 18 days after exposure (Swanson and Gillespie 1979, Lapeirre et al. 1988). After the first peak mortality, a second peak of mortality can occur a few days later (Reno, personal communication).

Host factors affecting IPN disease

Fish protect themselves against pathogens by both physical barriers and immune systems. If a pathogen manages to breach the initial physical barriers such as an epithelial shield of scales, skin, and also mucus, then serum factors such as serum lysozyme, lectins, interferon, complement, antibody and natural antibodies may directly inhibit or indirectly inhibit (such as opsonizing) it for further destruction (Fletcher, 1981).

The virulence of IPNV depends to a great extent on the particular host species and fish age. Although IPNV is regarded as a principal pathogen of salmonid fish, even within the salmonids there are marked differences between species in the degree of

susceptibility to IPNV. A few experimental challenges with IPN virus were carried out to determine its relative virulence for different species. The most susceptible species were brook and rainbow trout, whereas other salmonid species were less susceptible to lethal infection (McAllister and Owens, 1995; Hill, 1982).

In 1963, Parisot et al. reported that chinook salmon *Oncorhynchus tshawytscha*, Kokanee salmon *O. nerka* and coho salmon *O. kisutch* were resistant to the virulent isolates from brook trout. However, in 1973, Sano reported different results: that IPNV from rainbow trout was almost equally virulent for two *Oncorhynchus* species, amago *O. rhodurus*, and Kokanee salmon *O. nerka* and himemasu (landlocked form of *O. nerka*) as for rainbow trout fry.

In the case of Atlantic salmon, Swanson and Gillespie (1979) have reported that clinical IPNV failed to develop in young salmon fry experimentally infected by contaminated water with a rainbow trout isolate despite evidence of virus replication. Pancreatic necrosis without external clinical signs, however, was caused in this species by intraperitoneal inoculation of virus into yearling salmon. Both Atlantic salmon and brown trout can develop IPN disease and disease severity varies based on the virus strain (Hill, 1982). Even for a particular fish species such as rainbow trout, there may be differing degrees of susceptibility to different strains of IPN virus.

The age of the host is also an important factor in determining susceptibility to virus. Although there are some exceptions reported in older fish, susceptibility to IPN diseases steadily decreases with fish age. Fish older than 5-6 months can be infected by the virus, but they don't show clinical signs of disease even following intraperitoneal injection. Laboratory studies supported this age relationship (Franti and Savan, 1971;

Dorson and Torchy, 1981). Hill (1982) concluded that rainbow trout ceased showing signs of disease when 15-20 weeks old even though fish of this age may be actively infected by the virus following exposure. However, unusual mortality caused by an-IPN-like disease was reported from post-smolts of Atlantic salmon *Salmo salar* L. (Smail et al 1992). Later, it was found that the agent was IPNV-Sp. High mortality occurred within two weeks after smolts weighing 30-40g were transferred into sea cages. The fish did not show disease signs before the smolt stage and the virus titer from fish was low (10^4/g tissue) compared with the titers in trout succumbing to IPN disease (Reno 1976). Thus, it is assumed that older Atlantic salmon may be immune to IPNV, however the immune system was suppressed during the smolt stage.

To date, interferon, complement, and natural antibody are known to be important non-specific serum molecules inhibiting IPNV. Interferons (IFNs) are proteins or glycoproteins which are able to inhibit virus replication. Three types of IFNs (IFN-α, IFN-β, IFN-γ) are present in fish as well as in mammals. The first fish interferon was reported from fathead minnows cells by Gravell and Malsberger (1965). Thereafter, IFNs (α or β) were reported from teleost cell lines, including fin cells (GF) of blue striped grunt (Beasley et al. 1966), and fathead minnow cells (FHM) (Oie & Loh 1971). It is now well accepted that fish cells can secret interferon (α or β) after induction by viral infection (Kelly & Loh 1973, Dorson & de Kinkelin 1974, Snegroff 1993) or by exposure to poly I:C (MacDonald & Kennedy 1979, Eaton 1990). Interferon was induced in rainbow trout following infection with IPNV in 18 week old fry weighing 2.5 g (Dorson et al. 1992). IFN-γ-like molecules with antiviral activity and MAF activities were also detected when leucocytes from rainbow trout kidney were exposed to mitogens (Gram &

Secombes 1988, 1990). Interferon size in fish was reported mostly in 20-40 kDa range (Dorson et al. 1975, Tamai et al. 1993) although an unusual size was reported by De Sena & Rio (1975) where the size was 94 kDa. Interferon α or β are known to be stable at 56°C and pH 2 (Sano & Nagakura 1982). Tamai et al. (1993) purified IFN which was typsin sensitive and was fairly stable at the pH between 4 and 8. The antiviral activity retained about 60% of original activity at 60°C.

Virucidal activity of complement from rainbow trout and salmon has been reported (Sakai et al. 1994). The complement of trout is known to be inactivated when held at 40-45°C for 20 min. (Dorson et al. 1979, Sakai 1981, Ingram 1987,Røed et al. 1990). Although hemolytic activity of complement in rainbow trout fry (4-5 months after hatching) was not detected, complement in chum salmon *O. keta* fry (4-5 months after hatching) displayed virucidal activity against IPNV (Sakai et al. 1994). It was known that complement activity was decreased when fish were infected by gram-negative bacteria, *Aeromonas salmonicida* (Secomes & Olivier 1997). Also some individual complement molecules such as C3 can directly bind to virus and inhibit viral replication (Cooper & Nemerow 1986). Molecular weight of C3 in rainbow trout is approximately 190 kDa (Nonaka et al. 1981). C3 activation is dependant on the presence of Mg^{2+} (Pangburn & Müller-Eberhard 1980, Pangburn et al. 1981), and C3 binding is species selective (Huemer et al. 1993) and dependant on the cell line in which the virus has replicated (Hirsch et al. 1980, Hirsch et al. 1981).

Some serum antibodies in mammals are natural antibodies which are isotypes of IgM, IgG, or IgA produced by B cells lacking germline rearrangements or somatic mutations in the immunoglobulin coding regions (Baccala et al. 1989, Avrameas 1991).

Natural antibodies can react with proteins, but many of these antibodies can react with lipids or carbohydrates (Avrameas 1991). Natural antibodies show considerable antigenic cross-reactivity among different serotypes of virus and among different species of viruses (Welsh & Sen 1997).

Even though information on the presence of natural antibodies in fish and the significance of natural antibodies in resistance to viral infection is not well explained, two reports about natural antibody in fish are available. Natural antibody assumed to be IgM-like isotype was found in rainbow trout and these anti-TNP antibodies inhibited IPNV replication in RTG-2 cells (Gonzalez et al 1988, 1989). Therefore, the development and the concentration of these immune molecules are important factors in defense of fish against IPNV.

"6S" inhibitor in normal rainbow trout serum

It has been reported that an anti-IPNV inhibitor, called the "6S inhibitor", is present in rainbow trout serum (RTS) unexposed to IPNV (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974). Dorson & de Kinkelin (1974) reported that the serum inhibitor had a sedimentation coefficient of approximately 6S by ultracentrifugation and thus differs from fish antibody, IgM, which has a sedimentation coefficient of 14-16S. Not all virus isolates tested were inhibited by RTS, and the inhibition was not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Okamoto et al. 1983a, Ögut 1995). Ögut (1995) tested the RTS sensitivity of 109 IPNV isolates, from all

serogroup A serotypes (Hill & Way 1995): A_1=WB; A_2=Sp; A_3=Ab; A_4=He; A_6=Canada1; A_7=Canada2; A_8=Canada3; A_9=Jasper, and 65 IPNV isolates were inactivated (greater than 10^1 $TCID_{50}$/ml) by RTS. However, even within one serotype (A_1=WB), only 71% of virus isolates tested were inhibited by RTS.

It has been commonly reported that cell culture-adapted virus strains were more susceptible to RTS than were wild type virus (Dorson & de Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978). It was also reported that cell culture adapted virus was inhibited 50% by RTS at up to 0.01% concentration; however, wild type virus was not inhibited by 10% RTS (Dorson et al. 1978). Kelly & Nielson (1985) observed that viral adsorption to cells was inhibited in the presence of RTS. It has been reported that RTS sensitivity of IPNV developed sooner following multiple viral passages in a cyprinid cell line (EPC) than in the RTG-2 or BF cell lines (Hill & Dixon 1977). Hill and Dixon, (1977) reported that a naturally avirulent strain of IPNV in its 6S (after serial passing in the presence of NTS) resistant form did not induce antibody production. However, Hill (1982) suggested that generally virus in its 6S-sensitive stage does not induce neutralizing antibody in rainbow trout, while 6S-resistant virus does. However, it was suggested that the virus may need to have virulence factor itself in order to induce immunity.

It has been suggested by some researchers that the ability of IPNV strains to replicate in the presence of normal trout serum is an important aspect of virus pathogenesis and can be considered as a virulence factor (Hill & Dixon 1977, Hill 1982). However, 6S resistance does not always correlate with virulence and RTS does not always sustain viral virulence during viral passage *in vitro*. It has been reported that 6S

sensitivity was correlated with virulence of IPNV and that avirulent strains could become virulent by passaging virus in the presence of RTS (Hill and Dixon 1977, Hill 1982, Ögut 1995), although some researchers found opposite results (Dorson et al 1975, Dorson et al 1978, MacAllister and Owens 1986). MacAllister and Owens (1986) passaged IPNV VR-229 15 times in CHSE-214 cells in the presence (5% NTS in MEM) and absence (5% FBS in MEM) of RTS. Replicate groups of 50 fish (brook trout) were exposed to viruses passed 1, 5, 10 and 15 times in the presence and absence of NTS. It was found that virulence of virus was not conserved by passaging in the presence of RTS.

Virus factors affecting IPN disease

IPNV can cause mortality as high as 90-100%, especially in 1-4 month-old rainbow trout fry (Frantsi and Savan, 1971; McAllister, 1983). However, the mortality can be changed by the nature of virus itself as well as the health state of the host, and environmental factors. The virulence factors of IPNV have not been clearly determined.

A few researchers determined if plaque size was correlated with virulence (Dorson et al. 1978, Sano et al. 1992). However, although reassortment experiments demonstrated that although plaque size was encoded by genome segment A, which encodes VP2 and VP3 (Dorson et al. 1978, Sano et al. 1992), change in plaque size was not related to virulence (Sano and Okamoto, 1994).

Some researchers have determined the relationship between serotypes and virulence (Vestegård-Jørgensen 1971, Sano et al. 1992, MacAllister & Owens 1995, Ögüt

1995, Maret 1997). Although they found that virulent and avirulent isolates of IPNV were found within the same serotype, it is generally accepted that the Ab serotype (A3) isolates is avirulent for brook trout, whereas Sp and VR-299 serotypes are highly virulent (Vestegard-Jorgensen 1971). MacAllister and Owens (1995) found that the virulence was not associated with the species of host from which the virus was originally isolated. Maret (1997) examined whether epitopes were related to viral virulence. It was found that epitopes reacting with monoclonal antibodies directed against WB strain of IPNV were significantly related to mortality in young brook trout fry. Four of six epitopes related to virulence were found on VP2, and two on VP3.

Leavy et al (1994) indicated the ability of the virus to inhibit macromolecular synthesis by host cells can be an important virulence factor. Recently, it was found that IPNV induces apoptosis by down-regulation of survival factor Mcl-1 protein expression in a fish cell line (Hong JR et al. 1998, Hong JR et al. 1999). In CHSE-214 cells infected by IPNV, the Mcl-1 level markedly decreased during the first 8h postinfection, which resulted in cell death.

Glycosylation of proteins of enveloped viruses is an important factor in antigenicity (Caust et al. 1987). Although IPNV is not an enveloped virus, the possibility of glycosylation has been studied. The possibility of N-glycosylation in VP2 of IPNV has been suggested (Estay et al. 1990, Hávarstein et al. 1990) although contradictory results were also obtained (Perez et al. 1996, Nicholson, personal communication). Recently, Hjalmarsson et al. (1999) reported that VP2 of IPNV is not N-glycosylated but O-glycosylated. Espinoza et al (2000) suggested that VP2 is glycosylated in the cytoplasm. Thus, if IPNV is glycosylated, the cell line and the condition of cells in which virus is

replicated would be also important to the extent and configuration of viral glycosylation. It was also reported that a single amino acid substitution on a glycoprotein dramatically changed the virulence of rabies virus (Dietzschold et al. 1983).

INFECTIOUS PANCREATIC NECROSIS VIRUS

Virus replication

IPNV replicates in the cytoplasm and takes 16-20 hr at 22°C for one cycle (Malsberger RG, Cerni CP 1963). IPNV requires a low temperature - below 24°C - for replication, even though virion-associated RNA-dependent RNA polymerase works well in vitro at higher temperature (30°C) (Mertens et al 1982) and the viral mRNA can be faithfully translated in vitro at 37°C (Mertens & Dobos 1982, Duncan et al. 1987, Manning et al. 1990). In CHSE-214 cells at 4°C, it takes 2-3 hours for saturation of cellular binding sites (Dobos 1995). Although it has not been clearly determined how penetration or uncoating occurs, the virus is probably internalized within 20 minutes. The production of viral RNA was maximal at 8-10 hours postinfection, and is radically reduced by 14 hr (Somogyi & Dobos 1980). VP1 protein (VPg) is used as a primer which binds to 5' of each RNA strand (Dobos 1977).

Serological classification of aquatic birnaviruses

The serological classification of aquatic birnaviruses is complex. The possibility that IPNV is composed of multiple serotypes was first indicated by Wolf & Quimby (1971). Antibodies produced against isolate VR-299 incompletely neutralized two French isolates, which are now considered to belong to the Sp serotype (A_2) of Hill and Way (1995). Many birnaviruses have since been isolated from a variety of host species in different regions. These isolates were initially grouped into 3 serotypes, VR-299, Sp, and Ab), based on reciprocal neutalization titers with polyclonal antibodies (Leintz and Springer 1973, Macdonald & Gower 1981, Okamoto et al. 1983). More recently, Hill & Way (1995) tested nearly 200 isolates for serotyping by a standard reciprocal cross-neutralization test (Hill & Way 1995). They divided aquatic birnaviruses into 2 serogroups, A and B, in which no cross reaction by neutralization tests occurred but which reacted with each other by complement fixation. Serogroup A contained more than 200 isolates composed of nine serotypes: A_1 (Archetype WB), A_2 (Archetype Sp), A_3 (Archetype Ab), A_4 (Archetype He), A_5 (Archetype Te), A_6 (Archetype C1), A_7 (Archetype C2), A_8 (Archetype C3), A_9 (Archetype JA). Except for the two archetype strains, He strain (serotype A_4) from pike and Te strain (serotype A_5) from a marine bivalve mollusc, all of the archtype strains were originally isolated from salmonids suffering from IPN disease.

Serogroup B has only one serotype, B_1 (Archetype TV-1). To date, fewer than 10 isolates have been found from fishes and marine invertebrates in Europe (Hill and Way 1988b). Some of these isolates were determined to cause disease in trout (Ahne et al.

1989b); others were completely avirulent in salmonids and non-salmonids (Hill & Dixon 1977, Olsen et al. 1988).

Molecular biology of IPNV

Molecular biology of birnaviruses has not been intensively studied due to the lack of diseases in mammals (including humans) (Dobos 1995), which has resulted in slow progress. As the name "birnavirus" indicates, IPNV is composed of two segments of double-stranded RNA, segment A and B. The 5' termini of the viral nucleic acids is linked to a serine residue in the genome linked protein (VPg) by a phosphodiester bond (Calvert et al. 1991).

Segment A ($2.5x10^6$ Dalton), having 3097 bp in the Jasper isolate (Duncan & Dobos 1986), contains two open reading frames. The longer open reading frame encodes a 106-kDa polyprotein (NH2-preVP2-NS protease-VP3-COOH) which is cotranslationally cleaved by a viral protease (NS or VP4, 29 kDa) into pVP2 (62 kDa) and VP3 (31 kDa) (Duncan et al. 1987, MacDonald and Dobos 1981, Mertens and Dobos 1982). The pVP2 is further processed, during viral maturation, into VP2 which is major external protein and responsible for the reaction of type-specific neutalizing monoclonal antibodies (Dobos et al. 1977, Nicholson 1993). A universal, group-specific epitope has been reported to be located near the amino terminus of VP2, whereas the polypeptide responsible for a serotype-specific epitope has been mapped in the middle of the polypeptide (Dobos 1995). Heppell et al. (1995) reported that the central region of VP2

showed a more variable deduced amino acid sequences than its extremities. VP3 was thought to be an internal protein of the virus (Dobos and Rowe 1977) but at least a portion of VP3 is exposed on the surface since it reacts with a number of monoclonal antibodies (Caswell-Reno et al. 1986, Caswell-Reno et al. 1989, Nicholson 1993). VP3 showed comparatively stable sequences (Bruslind 1997, Nicholson unpublished data). Thus, a greater focus has been placed on VP2 than VP3 as the protein associated with the virulence of IPNV. However, a comparatively recent report indicated that VP3 contains at least one neutralizing epitope (Park & Jeong 1996).

The short open reading frame of segment A encodes a 17-kDa polypeptide. However, the presence of the polypeptide in the virion has not been proven, but identified only in infected cells (Duncan et al. 1991, Magyar and Dobos 1994). Harvarstein et al. (1990) detected a very faint band with the size of 17 kDa (called VP5) in purification of labeled IPNV; however, they could not confirm the identity of the protein due to the lack of specific anti-VP5 antiserum. Subsequently, Magyar & Dobos (Magyar & Dobos 1994) made monospecific rabbit antiserum against a purified 17 kDa protein. However, the antiserum did not react with the protein in western blots. Thus, it remains in question whether the 17 kDa peptide found by Havarstein et al. (1990) was in reality a small ORF product or a nonstructural polypeptide.

Segment B ($2.3x10^6$ daltons), with a size of 2784 bp in Jasper strain, encodes a few copies of internal polypeptide, known as VP1 (94 kDa), which is the putative RNA-dependent RNA polymerase (RdRp) (Poch et al. 1989). The VP1 can be present in two forms, a free polypeptide and a genome-linked protein or VPg (Calvert et al. 1991). Even though RdRp contains several conserved domains commonly found in other RNA

viruses, RdRp of IPNV has one unique characteristic; it lacks a Gly-Asp-Asp motif, which is common in this enzyme family (Gorbalenya & Koomin 1988, Duncan et al. 1991).

A few full length nucleotide sequences for segment A and B are available in the literature. Full sequences of segment A were determined for Jasper strain (A_9) (Duncan & Dobos 1986), Sp strain (A_2) (Mason 1992), and N1 strain (A_2) (Havarstein et al. 1990), while segment B has been sequenced for Jasper and Sp (Duncan et al. 1991). Both genome segments have noncoding regions at both ends, which is considered to be important for polymerase recognition, translation initiation and possibly genome packaging (Duncan et al. 1991). Heppell et al. (1995) sequenced the VP2 coding region of five IPNV strains (VR-299, Fr.21, Ab, C2, He) and compared them with three previously published strains: Jasper (Duncan & Dobos 1986), N1 (Hávarstein et al. 1990), and DRT (Chung et al. 1993). They revealed, through the comparison, that IPNV has a central variable domain (positions 183-335) which had two hydrophillic hypervariable segments. Ma (1996) suggested that IPNV-West Buxton might contain a subpopulation of neutralization-resistant viruses. These minor groups were resistant to neutralization by monoclonal antibody and had a very different nucleotide sequence compared to wild type parent virus. To investigate genomic variation, Heppell et al. (1993) sequenced 17 IPNV strains over a cDNA fragment located at the junction between pVP2 and NS coding regions. Although their sequences did not overlap with fragments encoding VP2 and VP3, they suggested the possibility that some of the 10 serotypes of IPNV could be subtypes because they found three genogroups. Nicholson (personal communication) has accumulated nucleotide and amino acid sequence information on

numerous IPNV isolates and found nine serotypes were present. Cutrin et al. (2000) also found a genetic diversity of IPNV isolated in Spain. They tested 231 strains of aquatic birnavirus isolated from fish, shellfish, and other reservoirs. Most of the isolates belong to European types Sp and Ab; however, 30% of the isolates could not be typed. Analysis of polyacrylamide gels showed six electopherogroups (EGs) in which 6.5% of the isolates showed the electropherotype characteristic of American strains (A_1).

DIAGNOSTIC METHODS

Cell culture methods and serological and biochemical methods are available to diagnose IPNV. Cell culture is the primary isolation technique, which depends on the production of cytopathic effect (CPE) of the cells. This is the most routinely used method because it is the most sensitive and stable technique. It was suggested that one or two infectious virions in 1ml of ovarian fluid (Amos 1985) or one virion in 10 liters of hatchery water can be detected by this method (Grinnell & Leong 1979, Watanabe et al. 1988). To date, over 30 continuous cell lines have been proved to be susceptible to aquatic birnavirus (Reno 1999) and the following cell lines are most often used: RTG-2 (rainbow trout gonad; Wolf and Quimby 1962); CHSE-214 (chinook salmon embryo; Lannan et al. 1984); FHM (fathead minnow; Gravell and Marlsberger 1965); BF-2 (bluegill fry; Wolf and Quimby 1966); EPC (epithelioma papulosum cyprini; Fijan et al. 1983). Each cell line has a different sensitivity to IPNV, and thus, at least two cell lines are required for diagnostic purposes (Hill 1976, Gillespie et al. 1977, Amos 1985). For

the isolation of aquatic birnaviruses in molluscs and invertebrates, BF-2 cell line was found to be the most susceptible (Hill 1982), while the CHSE-214 cell line was more susceptible than FHM and RTG-2 cell lines. If non-salmonids or shellfish are tested for the detection of IPNV, it is recommended to include a cell line from the homologous or closely related species. For example, the birnavirus isolated from the spinning disease of menhaden *Brevoortia tyrannus* could be detected only in cells from this fish species (MK cell line) (Stevens 1981).

Serological techniques combined with traditional cell culture techniques have been used for identification and classification of aquatic birnaviruses. The serum neutralization test using polyvalent antisera (Lientz & Springer 1973, Amos 1985) is the most common technique to confirm and to identify the virus. Other serological techniques for detection have been employed as follows; complement fixation (Finlay & Hill 1975), fluorescent antibody (Nicholson & Dunn 1974, Tu et al. 1974), immunoperoxidase tests (Reno 1976, Nicholson & Henchal 1978), neutralization kinetics (Nicholson & Pochebit 1981), *Staphylococcus* coagglutination (Kimura et al. 1984, Bragg & Combrick 1987a), counterimmunoelectrophoresis (Dea and Elazhary 1983), enzyme-linked immunosorbent assay (ELISA) (Nicholson & Caswell 1982, Dixon and Hill 1983b, Hattori et al. 1984), immunodot (Caswell-Reno et al. 1989, Ross et al. 1991), and immunoprecipitation (Lipipun 1988). However, these methods have drawbacks in the variation of sensitivity and detection limits, especially in detection from virus carrier fish. Therefore, these techniques should be used in combination with cell culture methods to overcome the handicap.

During the last 10 years, molecular techniques using nucleic acid probes have been developed. Oligonucleotide DNA probes have been used for direct detection of the viral genome. Dopazo et al. (1994) developed a dot-blot hybridization using cloned cDNA probes. However, it was found that cell culture technique should be combined to increase the sensitivity. The PCR techniques were developed to detect the viral genome. In practice, PCR can detect approximately 1 ng of genomic RNA (Cepica et al. 1991, Shankar & Yamamoto 1994); while a double-nested PCR assay (Rimstad et al. 1990) can detect 0.8 pg of ds-RNA, which is however, still less sensitive than cell culture. Thus, cell culture is still the best choice for diagnosis even though it takes more time than other techniques.

CHAPTER 3

BIOCHEMICAL CHARACTERISTICS AND ONTOGENY OF "6S" INHIBITOR OF AQUATIC BIRNAVIRUS IN NORMAL RAINBOW TROUT SERUM (RTS)

Kyoung C. Park and Paul W. Reno

ABSTRACT

In the present study, the characteristic of an inhibitor of infectious pancreatic necrosis virus (IPNV) found in normal rainbow trout serum (RTS) were investigated including molecular size, stability at different pH and temperatures, ontogeny in trout and effect of cations on the activity of the inhibitor. RTS inhibition of virus was obtained with molecules ranging from 50-300 kDa as measured by ultracentrifugation, sieve gel chromatography, and ultrafiltration, and the strongest inhibition was obtained at approximately 150 kDa as determined by all three methods. The inhibition capacity decreased significantly when RTS was dialyzed or filtered in the absence of divalent cations. RTS treated at temperatures ranging from 30 to 50 °C sustained its inhibitory activity at a level similar to non-treated RTS; however, treatment at 55°C completely destroyed the inhibitory capacity of RTS. With regard to the influence of extreme pH values, the inhibitory activity of RTS was not reduced between pH 4 and 10 but was diminished below pH 4 and above pH 10. Using pooled serum from normal rainbow trout, no inhibition was obtained until the fish had reached an age of 21 weeks post hatch and after 23 weeks post hatch, a RTS inhibitor continuously showed a significant level of inhibition.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is an aquatic birnaviruses which are most pervasive pathogens of teleosts as well as aquatic invertebrates (Reno 1999) and was the first virus isolated from teleosts (Wolf et al., 1960). Although IPN disease has been most commonly reported in trout, especially in young fry, IPNV can cause high mortality in a variety of non-salmonids as well (Reno 1999).

IPNV-inactivating activity in serum from fish which had not been exposed to aquatic birnaviruses has been reported from many sources. Antibody and interferon induction was well studied from IPNV-infected fish (Bootland et al. 1990, Dorson et al. 1992). It was also reported that natural antibodies were present in fish serum unexposed to IPNV. Gonzalez et al. (1988, 1989) reported the presence of natural serum antibodies in phylogenetically distinct fish species and that rainbow trout had anti-trinitrophenol antibodies, IgM-like antibodies (14-16S), which inhibited IPNV *in vitro*.

Although the identity of an inhibitor was not determined, since the early 1970's it has been reported that rainbow trout unexposed to IPNV had molecule(s) which inhibit IPNV replication *in vitro* (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974). This molecule appeared not to be antibody because that it had a sedimentation coefficient of approximately 6S by ultracentifugation, while fish antibody showed a sedimentation coefficient of approximately 14-16S (Dorson & de Kinkelin 1974). After the first report of "6S", several investigators have studied the phenomenon. However, those were mainly focused on RTS sensitivity of IPNV or the relationship between RTS sensitivity of IPNV and virulence (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974, Hill & Dixon

1977, Dorson et al. 1978, Ögut 1995). It has been shown that RTS sensitivity of IPNV is not consistently correlated with viral virulence (Hill & Dixon 1977, Hill 1982, Ögut 1995, Park unpublished results). Thus, the study of IPNV-infection is complicated due to the presence of a non-induced, non-immunoglobulin molecule with a 6S sedimentation coefficient in sera from rainbow trout not exposed to IPNV (Hill & Dixon 1977, Kelly & Nielson 1985). Consequently, if this "6S" molecule makes a significant contribution to the host defense against IPNV, its characterization is essential to the understanding of IPNV-related disease. Therefore, we conducted a series of experiments to determine the characteristics of this molecule and to determine the ontogeny of the inhibitory activity of RTS.

MATERIALS AND METHODS

Serum preparation

Rainbow trout were a kind gift of the Oregon Department of Fish and Wildlife hatchery at Alsea, OR. Prior and current fish health inspections indicate that there has been no IPNV detected at this facility for at least 25 years. Blood samples were collected from Alsea strain steelhead *Oncorhynchus mykiss* by caudal vein puncture and pooled from approximately 20 adult fish weighing approximately 600g each. The blood was allowed to clot at 5°C overnight and centrifuged at 1000×g for 20min. The serum was

collected and portions were dispensed into 1ml aliquots and stored in liquid nitrogen until use (Kelly & Nielsen 1985).

Virus

The IPNV isolate used was serotype A_9, subtype Jasper. This virus was isolated from diseased brook trout *Salvelinus fontinalis* in Maligne River Hatchery, Alberta, Canada (Yamamoto 1974), and was obtained from Dr. B. Nicholson, University of Maine, Orono, ME, U.S.A. and originally donated by Barry Hill (DAFF, Waymouth, U.K.). The virus had been passaged for 20 years in the laboratory. Prior to this experiment, the virus was passaged once in brook trout fry and two times in CHSE-214 cells. The virus was passaged two times through brook trout fry by immersion infection in $10^4 TCID_{50}$/ml water for 5h at 14°C, and once in CHSE-214 cells (Lannan et al. 1984).

***In vitro* virus inhibition by RTS inhibitor**

The RTG-2 cells (Wolf and Quimby 1962) used for this study were propagated in 24 well microtiter plates as described by Caswell-Reno et al (1989). Three replicate wells were prepared for each condition. In this experiment, three types of media were used: Eagle's Minimum Essential Medium (MEM) without serum=MEM-0; MEM+10% fetal bovine serum=MEM-10; MEM+10% fetal bovine serum+1%RTS=MEM-RTS. Virus

was diluted with MEM-10 or MEM-RTS to the virus concentration of $10^4 TCID_{50}$/ml. Three hundred μL of diluted virus were added onto a drained confluent monolayer in each well (0.01 M.O.I.) and incubated at room temperature for two hours. After the incubation time, the inoculated monolayers were washed three times with MEM-0 and then 1ml of either MEM-10 or MEM-RTS was added. Uninoculated controls were treated with either medium as appropriate. Cells were incubated at 18°C for seven days in an incubator to which 5% CO_2 was supplied. On the seventh day after exposure, cells were examined for the extent of cytopathogenic effect (CPE), cell culture supernatant from each well was harvested, and held in liquid nitrogen until they were titrated.

Virus titration

The endpoint dilution method as described in Caswell-Reno et al. (1986) was used for virus titration. Virus samples were serially diluted 10-fold with MEM-0 and then 100μL of each of diluted virus suspension were added to each of four wells of a 96 well plate containing monolayers of CHSE-214 cells. After incubation at 18°C for 7 days, wells showing CPE were counted to determine 50% tissue culture infectious dose ($TCID_{50}$/ml) (Spearman 1908).

Size determination of RTS inhibitor ("6S")

To determine the size of the inhibitor present in RTS, we employed three methods: ultracentrifugation, sieve gel chromatography, and ultrafiltration.

Ultracentrifugation: Rainbow trout serum (RTS) was fractionated by the modified method of Burke and Nisalak (1982). Briefly, 0.5ml of RTS was centrifuged through 4.5ml of discontinuous sucrose gradient (10-40% w/w) for 21 h at 32,000 rpm in a SW 50.1 Beckman rotor. Thirteen fractions (380 μL/each fraction) were collected by pipetting from the top of gradient. Three size markers (Sigma, St. Louis, MO) were ued for determining approximate size: Albumin from bovine serum (mol. wt: 66,000); Alcohol dehydrogenase from yeast (mol. wt: 150,000); β-Amylase from sweet potato (mol. wt: 200,000). To determine the position of marker proteins, each protein was ultracentrifuged and O.D. value at 280 nm was determined. Each fraction was diluted with MEM-10 to make the final concentration equivalent to a 1:100 dilution of RTS. The inhibition test of each fraction was conducted using the method described earlier for determining serum inhibition *in vitro*.

Ultrafiltration: RTS was fractionated by filters having pore size of 10, 30, 50, 100, and 300 kDa molecular cut off (Millipore, Bedford, MA). RTS was diluted to 1:10 with MEM-0, then 2ml of diluted RTS was added to each size of the filter-reservoir and was centrifuged at 1500-2500 x G. For obtaining of filtered size of RTS, the retentate was washed from the filter with TBS (10mM Tris-Hcl, 0.85% NaCl, pH7.4) containing 10mM $CaCl_2$ and 7.5mM $MgCl_2$, whereas the filtrate was passed through smaller pore size filters. Then samples were re-suspended in 1ml of MEM-0. The fractionated RTS was

diluted to 1:100 with MEM-10. Fractionated RTS was evaluated for inhibition activity against IPNV *in vitro* as described earlier.

Sieve gel chromatography: Rainbow trout serum was fractionated at 4°C in a column of Sephadex G-100 dextran gel (Sigma, St. Louis, MO) and was eluted in TBS (10mM Tris-HCl, 0.85% NaCl, pH7.4) buffer. Fractions of 1ml were collected and concentrations of protein in eluted fractions were determined by spectrophotometer at 280 nm with a Beckman DU-64 model. Three size markers (Sigma, St. Louis, MO) were used for determining approximate size: Albumin from bovine serum (mol. wt: 66,000); Alcohol dehydrogenase from yeast (mol. wt: 150,000); β-Amylase from sweet potato (mol. wt: 200,000). Three fractions were combined and each pool was filtered through a 0.2 μm filter and diluted to 1:10 in MEM-10. The inhibition test of each pool was conducted as described earlier.

Effect of divalent cations on the inhibitory activity of RTS

Dialyzer bag: one milliliter of RTS diluted (1:2) in MEM-0 was transfered into dialyzer tubing bag (VWR scientific, m.w. cutoff-12,000-14,000). RTS in the bag was dialyzed in 500ml of 10mM TBS containing 10mM $CaCl_2$ and 7.5mM $MgCl_2$ or in 500ml of 10mM TBS only. The bags were dialyzed three times in each buffer during 24 hrs at 5°C. The dialysate was then diluted to 1:100 in MEM-10 and filtered through 0.2μm filter. Non-dialyzed RTS (1:100 in MEM-10) and the two types of dialyzed RTS were tested for RTS inhibition activity as described earlier.

50K ultrafiltration: Diluted RTS (1:10) in MEM-0 was centrifuged through a filter having 50 kDa molecular cut, off then the retentate was washed 3 times with four types of buffer: 10mM TBS containing 10mM $CaCl_2$, or 7.5mM $MgCl_2$, or 10mM $CaCl_2$ and 7.5mM $MgCl_2$ or 10mM TBS only. Each washed 4 types of retained sera were diluted to the final RTS concentration of 1:100 as appropriate: for example, 1 ml of diluted RTS (1:10) in 10mM TBS containing 10mM $CaCl_2$ was mixed with 1ml of 10mM TBS containing 10mM $CaCl_2$ and 8 ml of MEM-10. RTS filtered and washed with 10mM TBS only was mixed with 1ml of 10mM TBS and 8 ml of MEM-10 to make 1:100 RTS dilution. As a positive RTS inhibition, RTS was directly diluted to 1:100 in MEM-10 without centrifugation. Diluted 5 types of RTS were filtered through 0.2 um filter, then used for RTS inhibition test as described earlier.

Temperatures stability of RTS inhibitor

Diluted RTS (1:10 in MEM-0) was incubated for 30 min in a water bath at temperatures ranging from 30°C to 70°C at 5°C intervals. After incubation, the samples were immediately cooled on ice and filtered through a 0.2 μm pore size filter. Control serum was preincubated at 5°C for 30min. The treated sera were diluted to 1:100 in MEM-10. After pretreatment of RTS, inhibition activity *in vitro* was tested as described above.

pH stability of RTS inhibitor

RTS was diluted to 1:10 in buffers having a pH ranging from 2 to 12. The following buffers were used: glycine-HCl, pH 2-3; acetate-acetic acid, pH 4-6; Tris-HCl, pH 7-9; glycine-NaOH, pH 10-12. Each treated RTS sample was incubated for 2 hr at room temperature and subsequently dialyzed three times against 10mM TBS (pH7.5,10mM $CaCl_2$ and 7.5mM $MgCl_2$). This treated serum was diluted again to 1:100 RTS in MEM-10 and filtered with 0.2 μm filter. The inhibition test of filtered RTS was conducted as the method of serum inhibition *in vitro* described earlier.

RTS inhibitor and fish age

In order to determine the ontogeny of the inhibitory activity of RTS, rainbow trout were bled and weighed from 5 weeks post-hatching to 42 weeks post-hatching at two week intervals. For a single time period, at least ten fish were sampled. The serum preparation from each bleeding was prepared by clotting and centrifugation as described above. Serum inhibition test *in vitro* was conducted with IPNV-Jasper. The inhibition test of each diluted serum was conducted as the method of described earlier.

RESULTS

Size determination of RTS inhibitor ("6S")

To determine the size of inhibitor in RTS, we employed three methods: ultracentrifugation, size-exclusion chromatography, and ultrafiltration.

Ultracentrifugation: RTS was fractionated by ultracentrifugation and fractions were tested for virus inhibition activity *in vitro* (Figure 3.1). When 13 fractions of RTS were assayed for anti-IPNV activity, significant inhibitions (greater than 10^{1} $TCID_{50}$/ml reduction) were found from fraction 3 with $10^{-2.8}$ $TCID_{50}$/ml (66 kDa marker position) to fraction 7 with $10^{-1.4}$ $TCID_{50}$/ml (200 kDa marker position). The strongest inhibition ($10^{-4.4}$ $TCID_{50}$/ml) was present in fraction 5 (150 kDa marker positioning). Even though the highest concentration of this marker protein (150 kDa) was obtained from fraction 5, the 150 kDa marker protein was also distributed at a low concentration from fraction 3 to fraction 7.

Ultrafiltration: RTS was fractionated by ultrafiltration (Figure 3.2). Inhibitory activity was present from 50K to 300K fractions. Before RTS fractionation, inhibition titer was $10^{-5.1}$ $TCID_{50}$/ml. After fractionation, maximum inhibition was obtained from 100-300 kDa, which showed $10^{-4.7}$ $TCID_{50}$/ml reduction. Fraction at 50-100 kDa also had a high inhibition, $10^{-2.7}$ $TCID_{50}$/ml reduction; however, the inhibition level was significantly lower than fraction 100-300 kDa. Other fractions showed insignificant levels of inhibition. MEM-10 containing 10mM $CaCl_2$ and 7.5mM $MgCl_2$, as a negative control, did not inhibit viral replication.

Sieve gel chromatography: Size-exclusion chromatography was performed on RTS to determine the approximate size of the viral inhibitor. As shown in Figure 3.3, significant inhibition (above 10^{-1} $TCID_{50}$/ml reduction) was obtained from fraction 43 to fraction 54. The fractions contained molecules with approximate molecular sizes from 150 kDa to 220 kDa. Fractions from 46 to 54, with approximate molecular weights from 150 to 200 kDa, showed the strongest inhibition ranging from $10^{-2.9}$ to $10^{-3.4}$ $TCID_{50}$/ml, while a fraction pool (43, 44, 45) having an approximate weight of 220 kDa showed low inhibition of $10^{-1.2}$ $TCID_{50}$/ml reduction. No significant inhibition was obtained from other fractions.

Effect of divalent cations on the inhibitory activity of RTS

To determine if RTS inhibitor requires cations for stability, we tested the effect of removing divalent cations, Ca^{2+} and Mg^{2+} by 50 kDa ultrafiltration or by dialysis RTS. Non-dialyzed RTS, as a positive control, had an inhibition level of $10^{-3.5}$ $TCID_{50}$/ml (Figure 3.4). However, when RTS was dialyzed without divalent cations the inhibition level was markedly decreased to 10^{0} $TCID_{50}$/ml. However, when RTS was dialyzed with divalent cations RTS showed a sustained inhibition level ($10^{-2.5}$ $TCID_{50}$/ml).

As shown in Figure 3.5, original RTS without 50 kDa filtration strongly inhibited IPNV ($10^{-6.7}$ $TCID_{50}$/ml). However, RTS retained on a 50 kDa filter without cations, such as Ca^{2+} or Mg^{2+}, had markedly decreased virus inhibition: 10^{-1} $TCID_{50}$/ml reduction

Figure 3.1 Distribution of activity of inhibition against IPNV-Jasper after fractionation on a discontinuous sucrose gradient (10-40% w/w) for 21 h at 32,000 rpm in a SW 50.1 rotor. Each fraction (0.3ml) was assayed against IPNV-Jasper (10^4 $TCID_{50}$/ml) on RTG-2 cells to determine inhibition activity. Arrows indicates the position of marker proteins: β-Amylase (200 kDa), Alcohol dehydrogenase (150 kDa), Albumin (66 kDa). The fraction numbers start from the top of tube.

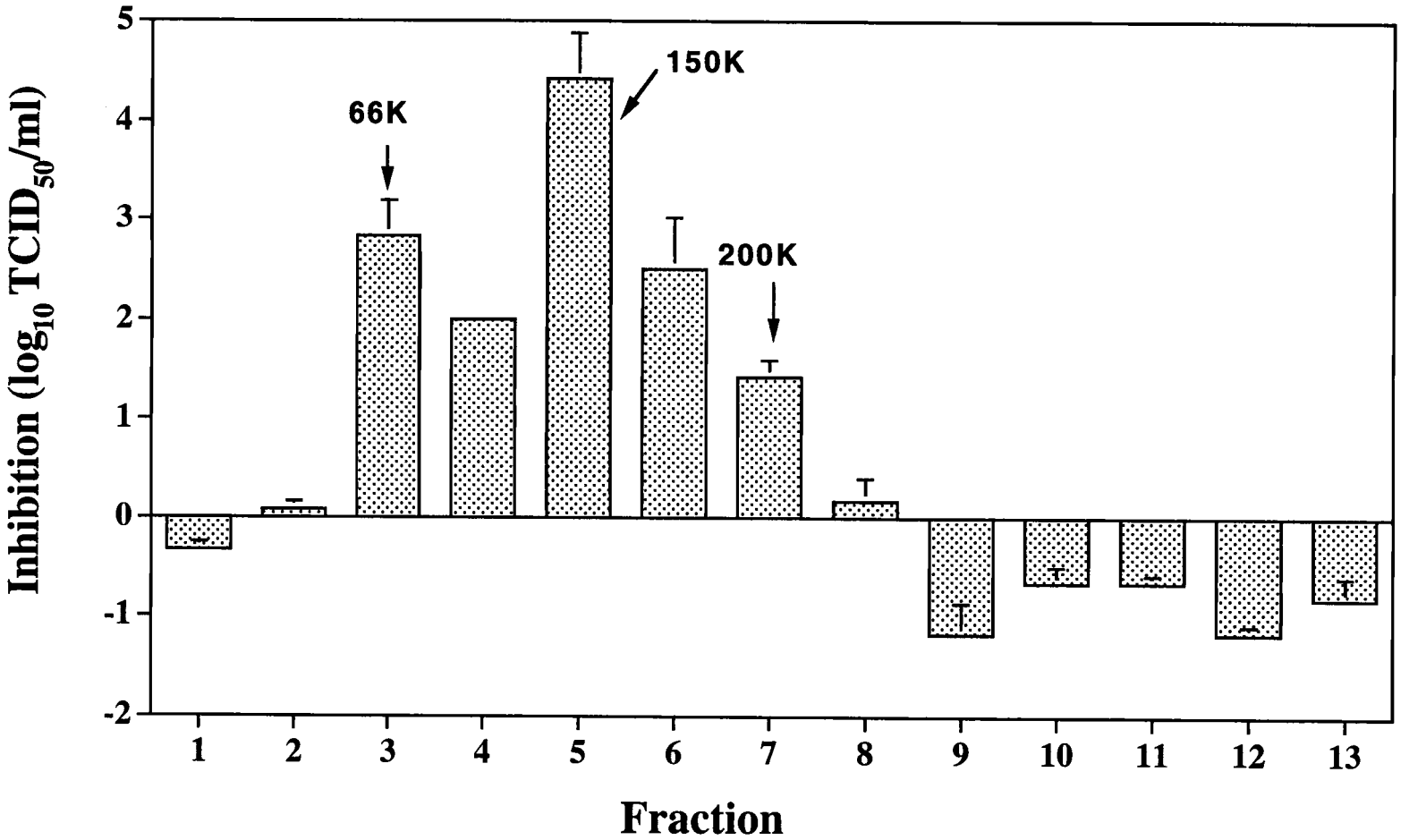
Inhibition (log_{10} $TCID_{50}$/ml)
5
4
3
2
1
0
-1
-2
66K
150K
200K
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction

Figure 3.1

Figure 3.2 Inhibition activity of RTS fractionated by filters having 10, 30, 50, 100, or 300 kDa pore-size. Diluted RTS (1:10 in MEM-0) was filtered through specific pore-size filters. For the size of 100-300 kDa, RTS was filtered with 100 kDa after a filtration of 300 kDa. The fractionated RTS was diluted to 1:100 in MEM-10 and inhibition activity *in vitro* was determined. MEM-10 (A) on X axis indicates an inhibition level in MEM-10 containing 10mM $CaCl_2$ and 7.5mM $MgCl_2$.

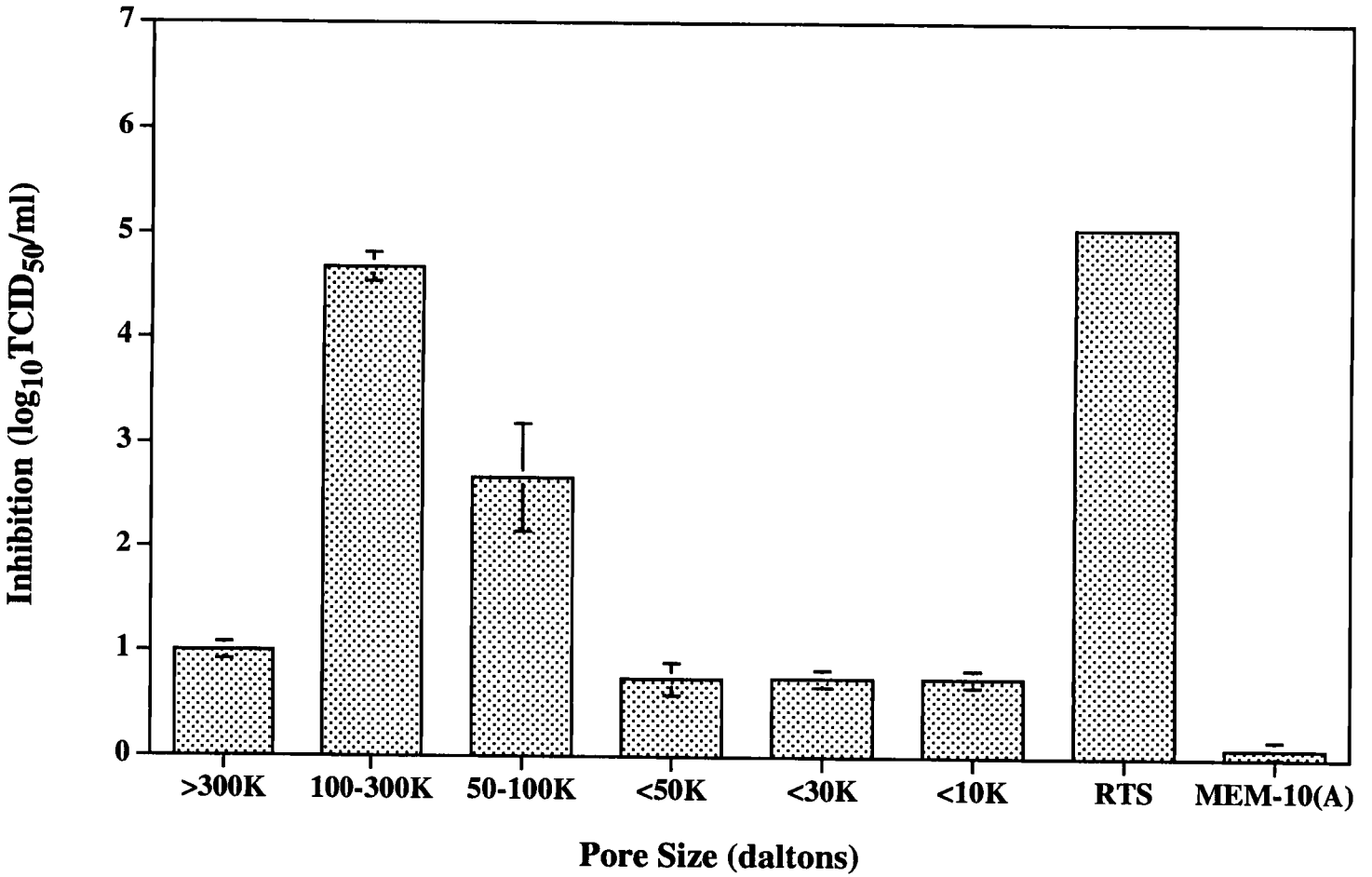
Inhibition ($log_{10}TCID_{50}$/ml)
7
6
5
4
3
2
1
0
>300K
100-300K
50-100K
<50K
<30K
<10K
RTS
MEM-10(A)
Pore Size (daltons)

Figure 3.2

Figure 3.3 Distribution of inhibition activity of RTS against IPNV-Jasper after fractionation on Sephadex G-100 dextran gel column having dimensions 65 by 2.0 cm. 1ml of RTS was eluted in a TBS (10mM Tris-HCl, 0.85% NaCl, pH7.4) buffer. Fractions of 1ml were collected and concentrations of protein in eluted fractions were determined by spectrophotometer. Three size markers (Sigma, St. Louis, MO) were used for determining approximate size: Albumin from bovine serum (mol. wt: 66,000); Alcohol dehydrogenase from yeast (mol. wt: 150,000); β-Amylase from sweet potato (mol. wt: 200,000). Three fractions were combined and each fraction pool was filtered with 0.2 μm filter and diluted to 1:10 in MEM-10. The RTS inhibition from each fraction pool was determined.

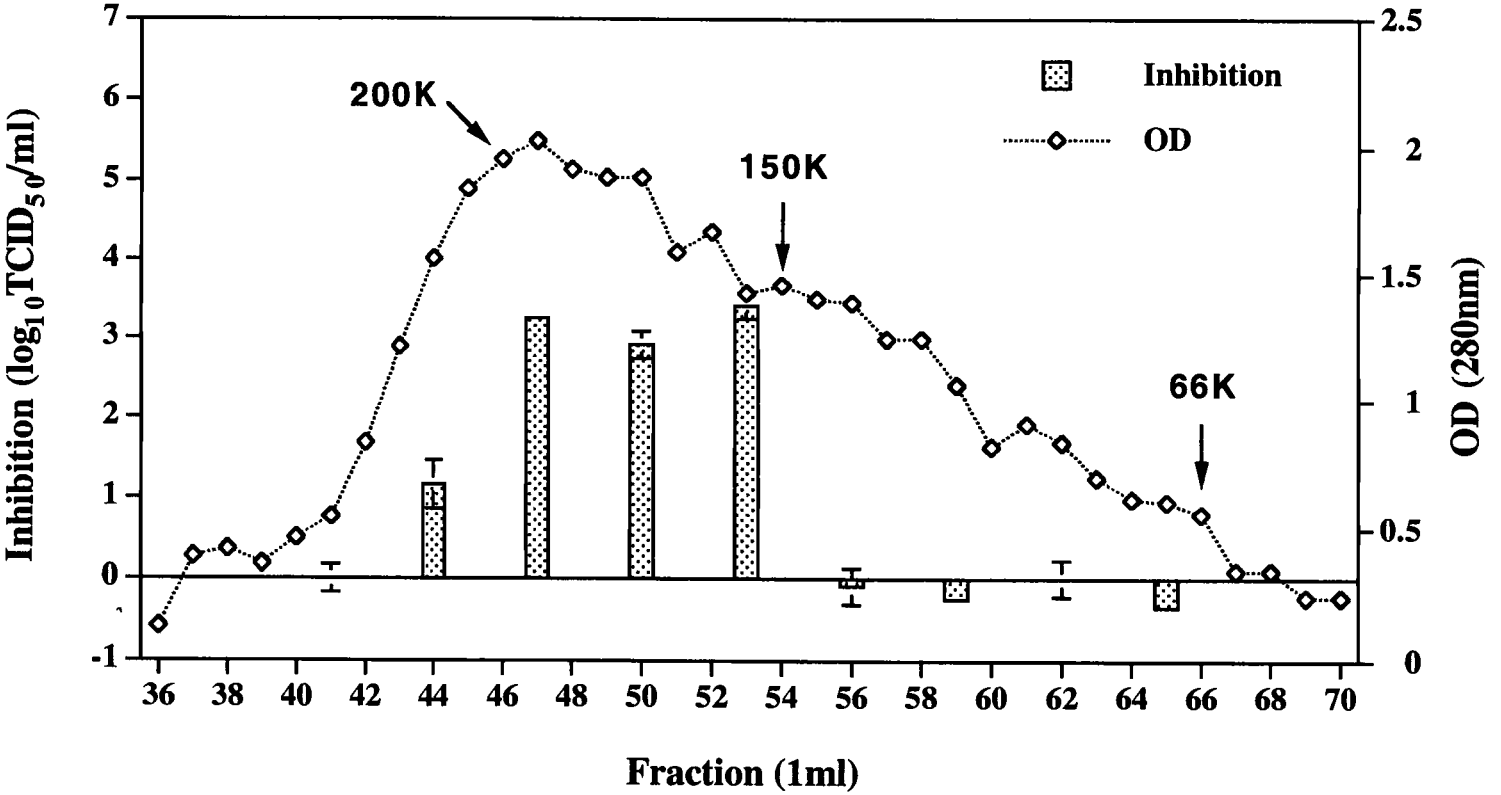
Inhibition
OD
200K
150K
66K
Inhibition ($log_{10}TCID_{50}$/ml)
OD (280nm)
Fraction (1ml)
7
6
5
4
3
2
1
0
-1
2.5
2
1.5
1
0.5
0
36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70

Figure 3.3

compared to control, MEM-10. The RTS, however, containing Ca^{2+} or Mg^{2+} or both cations retained RTS inhibition after 50K filtration even though inhibitory activity was somewhat decreased in comparison to the original inhibition level.

Temperatures stability of RTS inhibitor

The stability of the RTS inhibitor against IPNV at different temperatures was tested (Figure 3.6). RTS before treatment had an inhibition level of $10^{-4.6}$ $TCID_{50}$/ml. The inhibition activity was not affected by treatment at temperatures up to 50°C. However, treatment at temperatures greater than 50°C completely eliminated RTS inhibition.

pH stability of RTS inhibitor

The stability of the RTS inhibitory activity at different pH was tested (Figure 3.7). With regard to the influence of extreme pH values, the inhibition activity of RTS remained constant between pH 4 and 10 and diminished below pH 4 and above pH 10. The inhibition activity of RTS treated at pH values ranging from 4 to 10 was between $10^{-4.8}$ and $10^{-5.3}$ $TCID_{50}$/ml reduction. RTS inhibitor was more resistant to extremely high pH than extremely low pH: RTS treated at pH 11 and pH 12 showed minimally decreased inhibition ($10^{-4.4}$ and $10^{-4.3}$ $TCID_{50}$/ml reduction, respectively); however, RTS treated at

Figure 3.4 The effect of divalent cations, Ca^{2+} and Mg^{2+}, on the inhibitory activity of dialyzed RTS. RTS was dialyzed in 10mM TBS containing 10mM $CaCl_2$ and 7.5mM $MgCl_2$, or in 10mM TBS only. Both types of dialyzed RTS and non-dialyzed RTS (1:100 in MEM-10) were compared to determine the difference of virus inhibition activity.

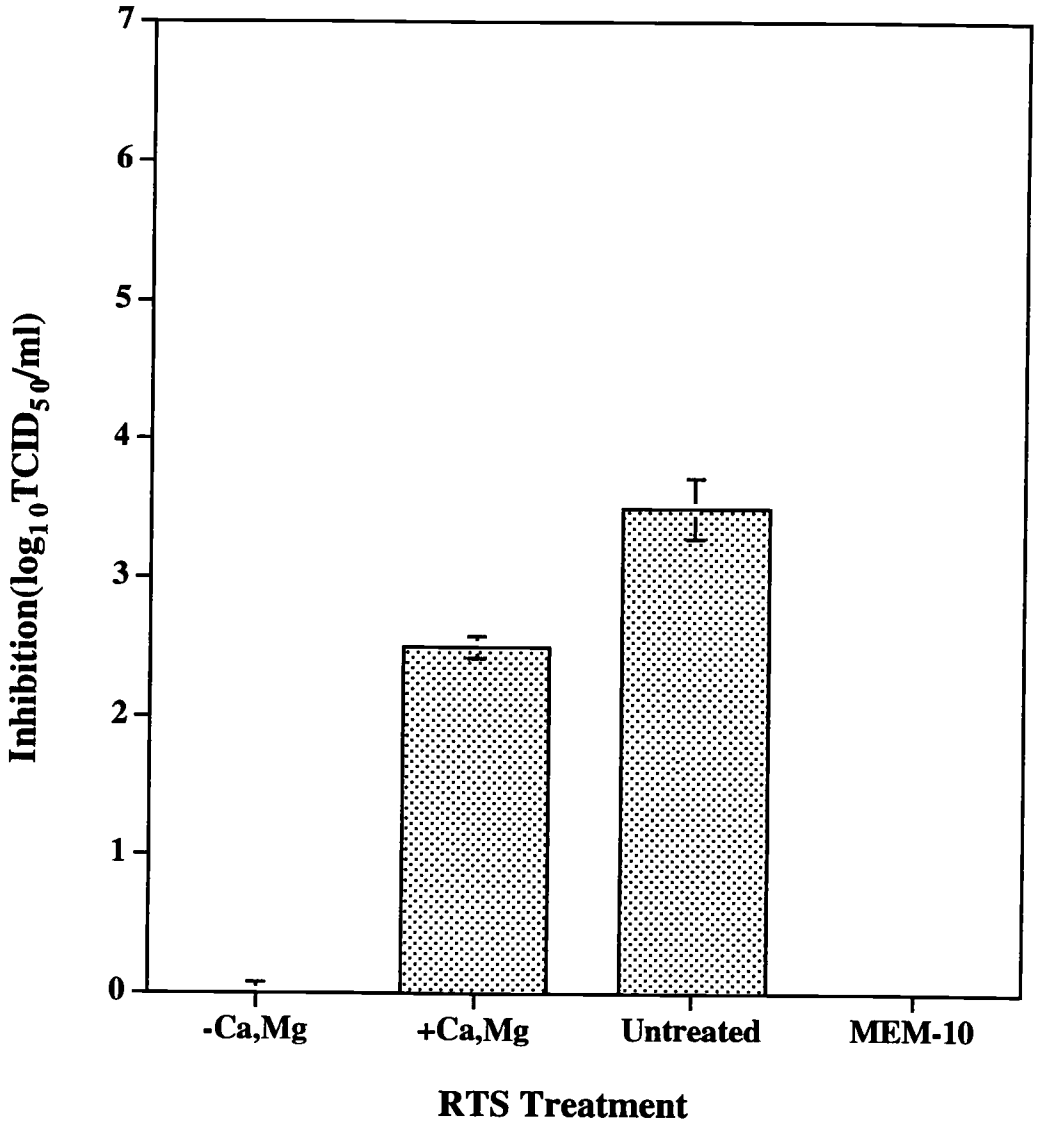
7
6
5
4
3
2
1
0
Inhibition(log10TCID50/ml)
-Ca,Mg
+Ca,Mg
Untreated
MEM-10
RTS Treatment

Figure 3.4

Figure 3.5 The effect of divalent cations, Ca^{2+} and Mg^{2+}, on the inhibitory activity of ultrafiltrated RTS. Components of RTS smaller than 50 kDa removed by a filter having 50 kDa pore-size. Retained RTS components were washed 3 times with TBS and diluted to 1:100 in four types of MEM-10 containing a final concentration of 1mM of $CaCl_2$, or 0.75mM of $MgCl_2$, or both 1mM of $CaCl_2$ and 0.75mM of $MgCl_2$, or no cation. RTS (1:100), a positive control, on X axis is the inhibition activity of RTS which was not filtered through 50 kDa pore-size. Inhibition activity *in vitro* was determined for each type of RTS.

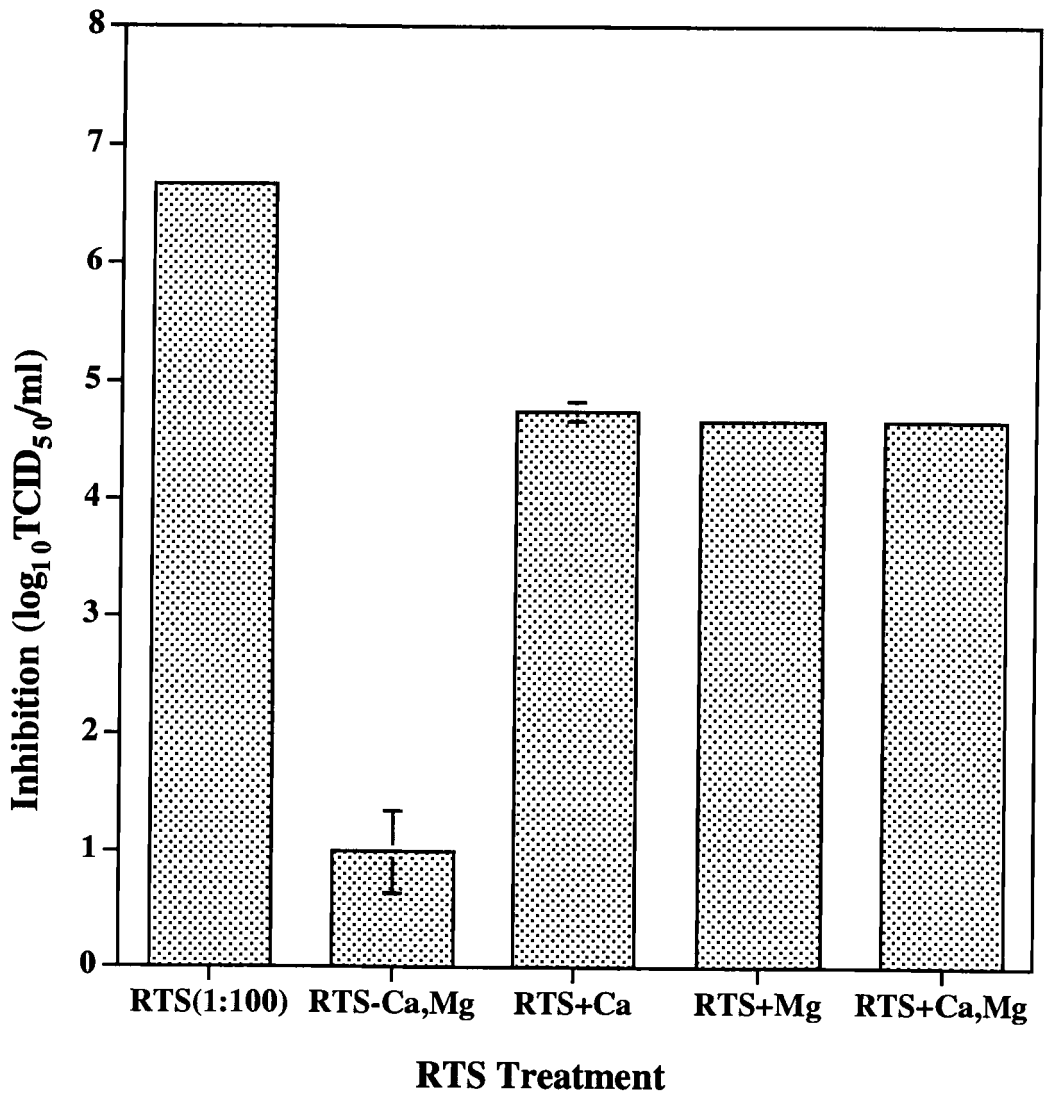
8
7
6
5
4
3
2
1
0
Inhibition ($log_{10}TCID_{50}$/ml)
RTS(1:100)
RTS-Ca,Mg
RTS+Ca
RTS+Mg
RTS+Ca,Mg
RTS Treatment

Figure 3.5

pH 2 or pH 3 showed a much reduced but still significant inhibition, $10^{-1.8}$ and $10^{-3.4}$ $TCID_{50}$/ml, respectively.

RTS inhibitor and fish age

We tested inhibitory activity from rainbow trout at various times after hatching in order to determine the relationship between fish age and level of inhibitor in RTS (Figure 3.8). At sampling intervals of two weeks with rainbow trout, no inhibition was noted until 21 weeks post hatch. At this time the inhibition level was low ($10^{-0.8}TCID_{50}$/ml reduction). After 23 weeks post hatch, however, RTS continuously showed significant inhibition (greater than $10^{-1}TCID_{50}$/ml) ($p<0.05$, compared to MEM-10). The smallest fish in which inhibition was found were in rainbow trout weighing approximately 2.39g. This inhibition was lower than the positive control inhibition of virus obtained for adult rainbow trout serum of $10^{-3.5}TCID_{50}$/ml reduction in virus titer.

Figure 3.6 Stability of vius inhibitory activity of RTS at different temperatures. RTS (1:10 in MEM-0) was incubated for 30 min. in water bath at temperature ranging from 30°C to 70°C. Control serum was incubated at 5°C for 30 min. MEM on X axis is a negative control, (MEM-10), for RTS inhibition. Treated RTS were diluted to 1:100 in MEM-10 and were tested for serum inhibition *in vitro*.

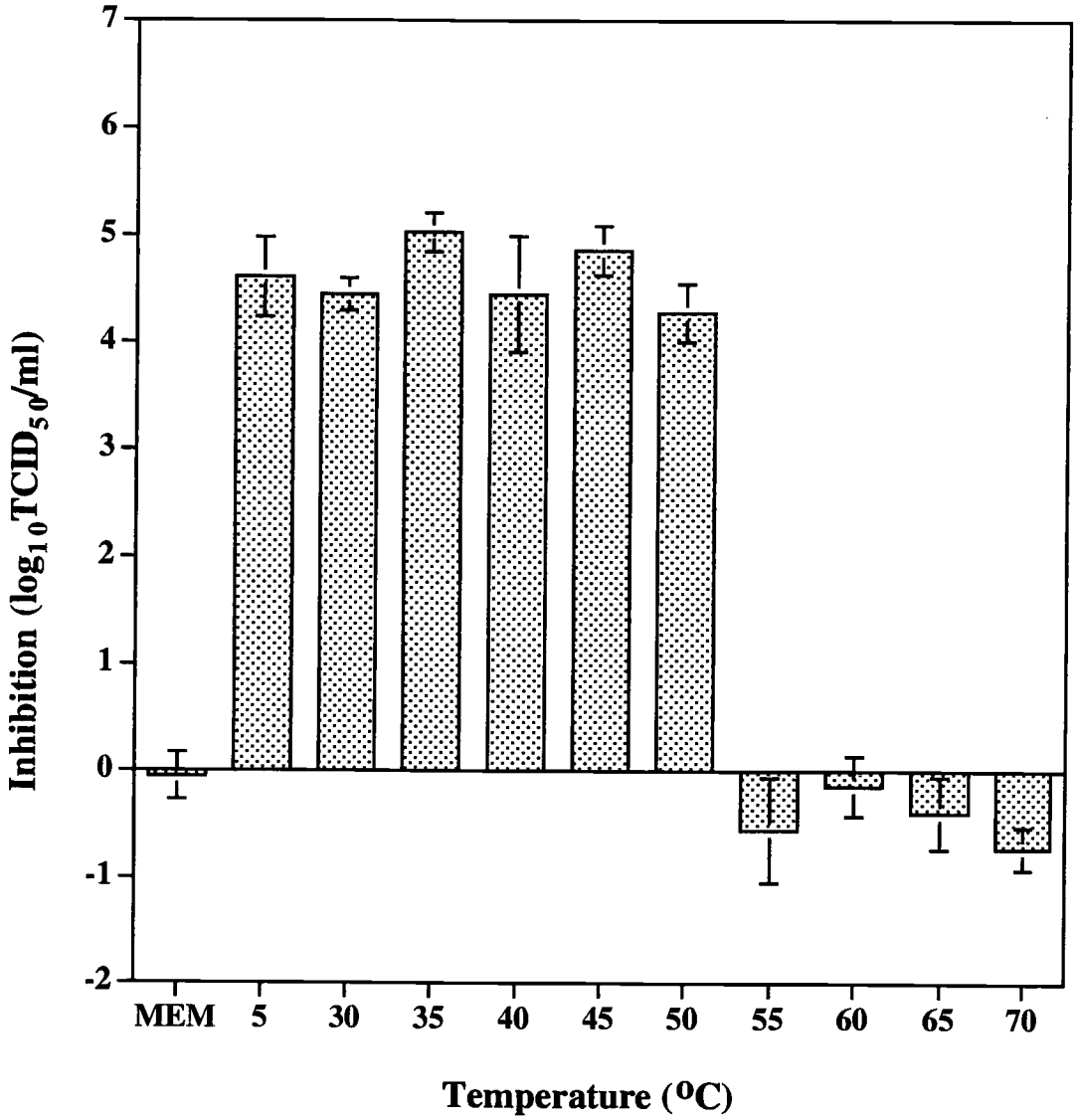
7
6
5
4
3
2
1
0
-1
-2
Inhibition ($log_{10}TCID_{50}$/ml)
MEM
5
30
35
40
45
50
55
60
65
70
Temperature (^{o}C)

Figure 3.6

Figure 3.7 Stability of virus inhibitory activity of RTS at various pH. RTS was diluted to 1:10 in buffers having different pH ranges from 2 to 12. The following buffers were used: glycine-HCl, pH 2-3; acetate-acetic acid, pH 4-6; Tris-HCl, pH 7-9; glycine-NaOH, pH 10-12. Each was incubated for 2 hr at room temperature and subsequently dialyzed three times with 10mM TBS (pH 7.5, 10mM $CaCl_2$ and 7.5mM $MgCl_2$). The treated RTS (1:100 in MEM-10) were tested for RTS inhibition *in vitro*.

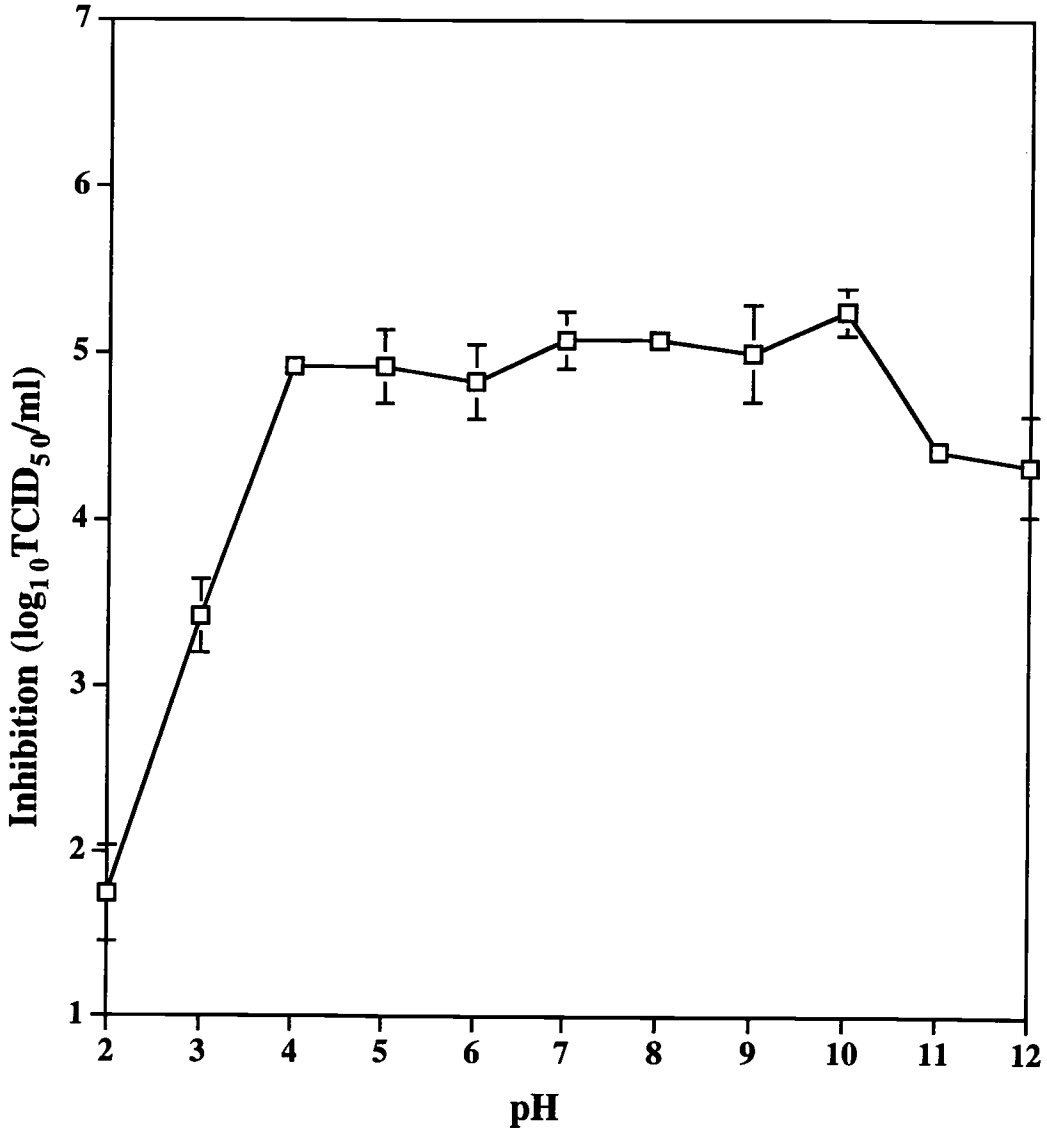

Figure 3.7

Figure 3.8 The development of inhibition activity of RTS against IPNV-Jasper after hatching. RTS was obtained at two weeks intervals from 5 weeks post-hatching fry to 42 weeks post-hatching rainbow trout fry. Second Y axis shows average fish weight (g) at each week after hatching. The inhibition activity from each serum (MEM-RTS) was conducted on RTG-2 cells. Inhibition of virus titer between MEM-RTS and MEM-10 was measured 7day postexposure.

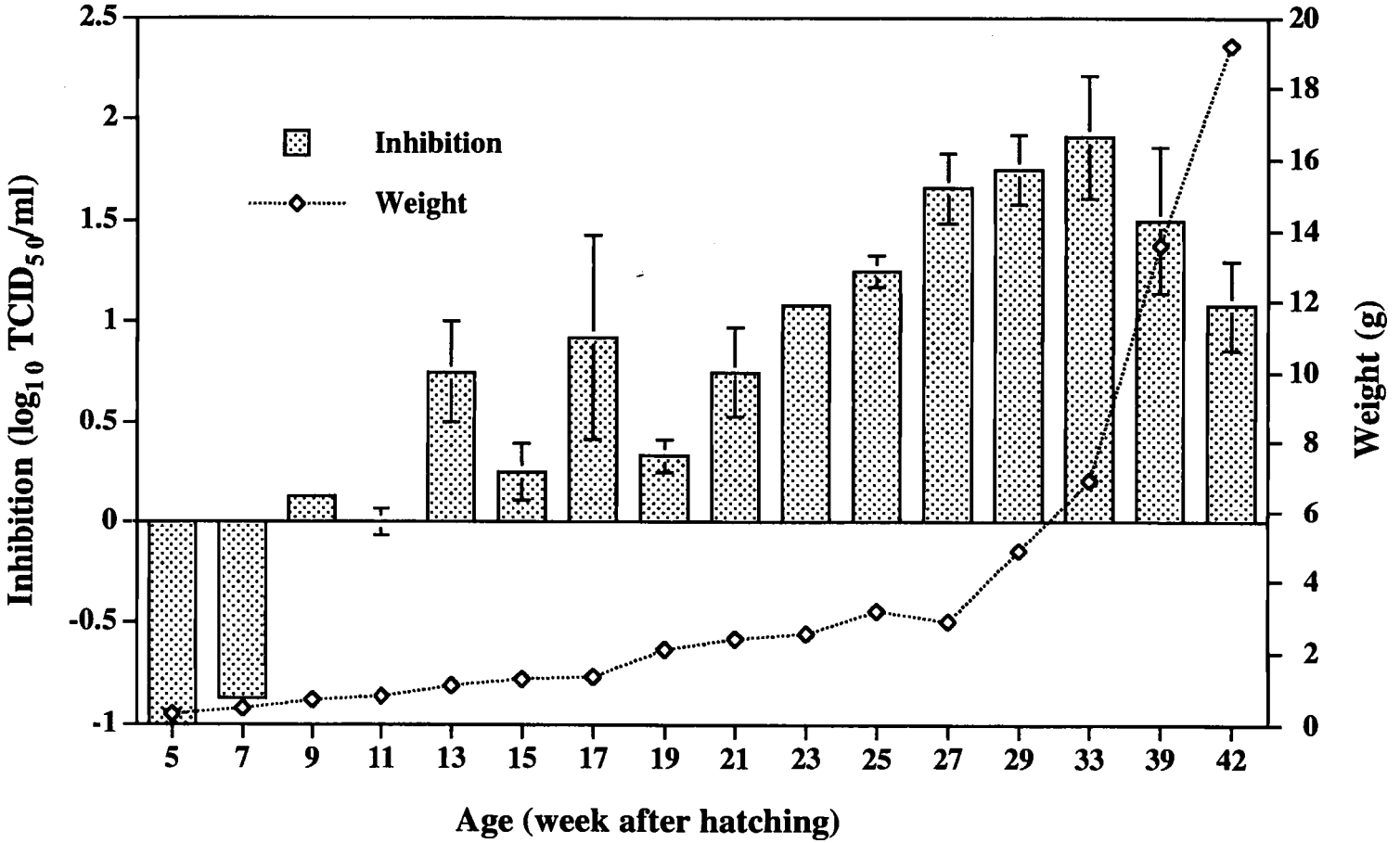
Inhibition
Weight
Inhibition (log10 TCID50/ml)
Weight (g)
Age (week after hatching)
2.5
2
1.5
1
0.5
0
-0.5
-1
20
18
16
14
12
10
8
6
4
2
0
5
7
9
11
13
15
17
19
21
23
25
27
29
33
39
42

Figure 3.8

DISCUSSION

This study demonstrates the characteristics of RTS inhibitor ("6S") against IPNV. These and other results (Park unpublished results) allow us to narrow the possible nature of inhibitory molecules to a "6S" inhibitor in RTS.

Complement and interferon have been well studied in terms of nonspecific humoral defense molecules in fish against IPNV (Sakai et al. 1994, Dorson et al. 1992). Even though natural antibody is not a humoral defense molecule, it was also often mentioned as a potential nonspecific defense molecule against IPNV (Gonzalez et al. 1989). It is well known that lectins can agglutinate bacterial pathogens of fish (Yano 1996), even though no information is available that lectin can inhibit viral pathogen of fish. However, it was often reported that serum lectins inhibited virus such as HIV-1 (Ezekowits 1989) or Inflenza A virus (Hartley et al. 1992).

Even though "6S" is not an inducible molecule (Park, unpublished results) and does not have not characteristics corresponding to antibody, it should be proved, at first, that "6S" inhibitor is not antibody. Although salmonids were thought to have only a tetrameric IgM-like structure when "6S" inhibitor was first reported, Sánchez and Domínguez (1991) showed the existence of size heterogeneity in rainbow trout immunoglobulin, probably corresponding to tetramers, trimers, dimers, monomers at 700, 540, 360, 180 kDa. However they found that the majority of the molecules are 700kDa, while other size molecules were in the minority. We found RTS inhibition was highest at the size of approximately 150 kDa, and the significant level of inhibition was shown also at the size between 50-100 kDa (Figure 3.1, 3.2). Ögut (1995) also reported that even

within one serotype (A1=WB), not all virus isolates tested were inhibited by RTS. This is not characteristic of antibody. It was also reported that RTS sensitivity was dependent on cell lines in which virus was replicated (Hill & Dixon 1977, Park unpublished results). Thus, RTS inhibition is very unlikely to be an anti-IPNV antibody. It was, however, also reported that natural antibodies were present in fish serum unexposed to IPNV. Gonzalez et al. (1988, 1989) reported the presence of natural serum antibodies in phylogenetically distinct fish species and that rainbow trout had a natural anti-trinitrophenol antibody which inhibited IPNV *in vitro*. However, the results in this paper indicate that RTS inhibitor may not be a natural antibody. Natural antibody in fish is considered to be an IgM-like molecule (Gonzalez et al. 1988). If RTS inhibitor is natural antibody, inhibition should be found at the size above 300kDa. We found insignificant inhibition above 300 kDa, but also found significant inhibition between 50-100K kDa (Figure 3.2). Thus, we suspect that the RTS inhibitor is between 66K and 150K.

We considered the possibility of the inhibitor being interferon because the size of interferon was reported as approximately 94 kDa (de Sena & Rio 1975), although interferon size in fish was reported mostly in 20-40 kDa (Dorson et al. 1975, Tamai et al. 1993). Another reason that we suspected interferon was that RTG cells and FHM cells, which have been used in RTS inhibition tests, produce interferon (Gravell & Malsberger 1965, Oie & Loh 1971, de Kinkelin & Dorson 1973). Interferon α or β are known to be stable at 56°C and pH 2 (Sano & Nagakura 1982). But RTS inhibitor, in our experiments, was labile at those conditions (Figure 3.6, 3.7). Interferon is known to be susceptible to these conditions and to trypsin (Pestka & Baron 1981, Sano & Nagakura 1982). However, we found RTS inhibition activity was stable after treatment with trypsin or

proteinase K (data not shown). Interferon does not require metal ions for function or stability. The results reported here clearly indicate that inhibitory activity is dependent on divalent cations, with either Ca^{++} or Mg^{++} alone sufficing to support activity. In our previous report (Park unpublished results), we found RTS inhibitor directly affects IPNV before viral penetration rather than protecting the cells. Thus, the data presented here strongly suggest that RTS inhibitor is neither natural antibody nor interferon. Nor is it likely to be specific antibody since IPNV has not been detected in the hatchery from which the fish were obtained.

Virucidal activity of complement from rainbow trout and salmon has been reported (Sakai et al. 1994). Complement activity was decreased when fish infected by Gram-negative bacteria, *Aeromonas salmonicida* (Secomes & Olivier 1997). In our labotatory, Rainbow trout were injected intraperitoneally with *V. angullarum* or PBS to determine if RTS inhibitor against IPNV is an inducible molecule by Gram-negative bacteria having LPS. RTS inhibition was not induced by the bacteria (data not shown). Although the endpoint of RTS inhibition was not changed, we found a difference between control serum from PBS injection and the serum from *V. angullarum* injection at the endpoint of RTS inhibition, 1:800 dilution. The RTS injected with *V. angullarum* showed a reduced RTS inhibition compared to control RTS. Thus RTS inhibitor is not an inducible molecule but can be reduced by Gram-negative bacteria. The complement of trout, however, is known to be inactivated when held at 40°C-45°C for 20 min. (Dorson et al. 1979, Sakai 1981, Ingram 1987,Røed et al. 1990). In our tests (Figure 3.6), RTS inhibition was stable up to 50°C. When RTS was treated with zymosan, a complement activator, inhibition activity of RTS was not changed (data not shown). Therefore, the

data do not support the likelihood of a member of the complement complex as RTS inhibitor; however it is known that each complement molecule such as C3 can directly bind to virus and inhibit viral replication (Cooper & Nemerow 1986). Molecular weight of C3 in rainbow trout is approximately 190 kDa (Nonaka et al. 1981). Although RTS inhibition was found at the wide size range between 50-300 kDa (Figure 3.1, 3.2), most stable and highest inhibition was obtained around 150 kDa which overlaps with the size of C3. The activation of C3 is also depend on the presence of Mg^{2+} (Pangburn & Müller-Eberhard 1980, Pangburn et al. 1981). C3 binding is species selective (Huemer et al. 1993) and also dependant on cell line in which virus is replicated (Hirsch et al. 1980, Hirsch et al. 1981). We also found in other experiments (Park unpublished results) that inhibitory activity against IPNV was found only in salmonid serum and was dependant on cell line. Thus the possibility of inhibition by C3 still remains viable.

We found that RTS inhibitor was more stable in the presence of cations than in the absence when it was fractionated or partially purified (Figure 3.4, 3.5). Some molecules are stable in the presence of ions when purified. Some serum molecules, such as some types of lectins, require ions for stability or function (Drikhamer 1988). Lectins in fish serum were purified in the presence of calcium (Gercken et al. 1994, Holmskov et al. 1994, Jensen et al. 1997). Some lectins require calcium for binding and for stability. Specifically, calcium is required when the molecule is a multimeric form for folding and stability (Kawasaki et al. 1987). We found that RTS inhibitor was positioned from 50-100K fraction to 300K even though the highest inhibition was obtained around 150K. Additionally RTS inhibition was stronger when it was partially purified with presence of cations than without cations (Figure 3.4, 3.5). On the other hand, the inhibition activity of

RTS was stable at temperatures up to 50°C and at pH between pH 4 and 10 (Figure 3.6, 3.7). This information on the stability of RTS inhibitor at different pH and temperature indicates that 6S inhibitor is physiochemically stable in harsh conditions. Lectin is also one of the serum molecules which are resistant to relatively extreme conditions of pH or temperature. A mannan binding lectin (MBL) isolated from serum of *Anguilla anguilla* is stable between pH 4 and pH10 and temperatures below 55°C (Gercken and Renwrant, 1994). In a preliminary experiment (data not shown), we found that some carbohydrates, mannan and n-acetyl-D-galactosamine, reduced the RTS inhibition activity when RTS was preincubated with these carbohydrates.

We still question whether RTS inhibitor is a lectin because lectins bind to carbohydrates. However, IPNV is not an enveloped-type virus and conflicting results about glycosylation of IPNV have been obtained (Dobos 1995): the possibility of glycosylation in IPNV has been suggested (Estay et al. 1990, Hávarstein et al. 1990, Hjalmarsson et al. 1999); contradictory results, however, were also obtained (Perez et al. 1996, Nicholson personal communication).

Even though more work should be done to determine if IPNV is glycosylated, our results corresponded more with the characteristics of lectins than other serum molecules. Therefore, it will be worth to study the effects of carbohydrates on RTS inhibition activity against IPNV to identify the identity of RTS inhibitor which is known as "6S" RTS inhibitor.

While the *in vitro* inhibitory activity of RTS is of interest, its *in vitro* function should also be addressed. It is obvious that the ability to inhibit the replication of IPNV can bolster survival, and details of this activity remain unexplored. It is well known that

IPN disease predominantly affects fry & fingerling trout (Wolf 1988). We found first inhibition at 23 weeks post-hatch (Figure 3.8) when RTS was tested at a dilution of 1:100. Thus, inhibition probably starts earlier than 23 weeks post-hatch if undiluted RTS was tested. Dorson & Torchy (1981) also noted the influence of fish age on mortality of rainbow trout fry caused by IPNV. The fry showed a decrease in sensitivity with increasing age and ceased to be susceptible to the disease at all when 20 weeks old. The age at which rainbow trout show resistance to IPN disease is closely related to the age at which first IPNV inhibition appears. Interferon was found to be synthesized by rainbow trout following infection with IPNV at the age of 18 weeks post-hatch when the mean weight was 2.5 g (Dorson et al. 1992), while first IPNV inhibition in our experiment (Figure 3.8) was obtained at 23 weeks post-hatch with the mean weight of 2.4g. This may suggest that the non-specific immune system against IPNV develops at this age. Bootland et al. (1990) found that trout fry were protected against IPNV only when they were immunized during the time of slow weight gain. We also found that RTS inhibition was decreased at the time of 39 and 42 weeks post-hatch when mean weight of rainbow trout fry was rapidly increasing (Figure 3.8). Therefore, fish weight gain rates seems to be an important factor for protection against IPNV and the RTS inhibitor appears to be an important non-specific immune molecule to resist IPNV.

In conclusion, we found that IPNV inhibitor, "6S", ranges in molecular weight between 50-300 kDa (most abundant at approximately 150 kDa) and requires divalent cations, such as Ca^{2+} or Mg^{2+}, for stability or function. RTS inhibitor was stable enough to resist treatment at temperature up to 50°C and pH between 4-10. RTS inhibitor appears

to be important non-specific immune molecule to resist IPNV and was observed after 23 weeks post hatch of rainbow trout weighing approximately 2.39g.

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CHAPTER 4

THE INHIBITION MECHANISM OF RAINBOW TROUT SERUM (RTS) INHIBITOR AND THE CHARACTERISTICS RELATED TO RTS INHIBITORY ACTIVITY

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ABSTRACT

The mechanism of rainbow trout serum (RTS) inhibition and characteristics of RTS inhibitor against infectious pancreatic necrosis virus (IPNV) were studied. The pretreatment of cells with RTS did not induce inhibition of IPNV and thus did not involve masking a viral receptor. The RTS inhibition level was dependent on the time at which virus was exposed to RTS, with inhibition maintained for at least 16h postinfection. Pretreatment of IPNV indicated that virus is directly inhibited by RTS and more strongly inhibited when RTS is present during viral replication. Serum inhibition is related to serum source and host cell specificity. IPNV was more efficiently inhibited by RTS in salmonid cell lines than in non-salmonid cell lines and inhibition level was highest in RTG-2 cells. RTS sensitivity of virus was altered by the cell line in which virus was passaged, since multiple passages in CHSE-214 produced virus which was less sensitive to RTS. RTS inhibition level was dependent on cell density: at a cell density $\leq 2x10^5$/ml, inhibition was insignificant ($10^{-1.1}TCID_{50}$/ml reduction); however, above a density of $3x10^5$/ml, the inhibition level was very high ($\geq 10^{-6.3}$TCID50/ml reduction). Salmonid sera tested showed high inhibition, except for brook trout serum (BTS), while non-salmonid sera did not inhibit IPNV replication on RTG-2 cells.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV), belonging to the family birnaviridae, causes highly destructive diseases of non-salmonids as well as salmonid species (Reno 1999). It has been reported that anti-IPNV inhibitor, called "6S inhibitor", is present in rainbow trout serum (RTS) unexposed to IPNV (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974). Dorson & de Kinkelin (1974) reported that the serum inhibitor had a sedimentation coefficient of approximately 6S by ultracentrifugation and thus differs from fish antibody, IgM, which has a sedimentation coefficient of 14-16S. Since the first report of IPNV inhibition by RTS, many reports have been published. However, most of those reports were limited to information about RTS sensitivity of IPNV or the relationship between RTS sensitivity of IPNV and virulence (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978, Ögut 1995). Not all virus isolates tested were inhibited by RTS, and the inhibition was not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Okamoto et al. 1983a, Ögut 1995). It was reported that cell culture adapted virus was inhibited 50% by RTS at dilution of 0.01% concentration; however, wild type virus was not inhibited even by 10% RTS.

It has been commonly reported that cell culture-adapted virus strains were more susceptible to RTS than were wild type virus (Dorson & de Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978). It has been also reported by some researchers that RTS sensitivity was correlated with virulence of IPNV and was changed by cell passage (Hill and Dixon 1977, Hill BJ 1982, Ögut 1995). At present, however, only a few reports are available that related to the mechanism of RTS inhibition or characterization of RTS

inhibitor. Kelly & Nielson (1985) observed that viral adsorption to cells was inhibited in the presence of RTS. It was reported that RTS sensitivity of IPNV was developed sooner in the case of multiple viral passages in EPC cyprinid line than in the RTG-2 or BF cell lines (Hill & Dixon 1977). Even though it was not mentioned that RTS inhibition ("6S") was related with cellular induction by serum molecules such as interferon, some reports showed that IPNV was inhibited by serum molecules such as interferon (de Kinkelin & Dorson 1973; de Kinkelin & Le Berre 1974; Dorson et al. 1992). Therefore, a series of experiments was conducted to determine the inhibition mechanism of RTS inhibitor of IPNV and the characteristics related to RTS inhibitory activity.

MATERIALS AND METHODS

Serum preparation

Fish were obtained either from wild or hatchery sources. Detailed information about fishes used as serum sources is listed in the Table 4.1. Rainbow trout were obtained from the Oregon Department of Fish and Wildlife hatchery at Alsea, OR. Prior and current fish health inspection indicates that there has been no IPNV detected at this facility for more than 25 years. Blood samples were collected from Alsea strain steelhead (*Oncorhynchus mykiss*) by caudal vein puncture and pooled from approximately 20 adult fish weighing approximately 600g each. The blood was allowed to clot at 5°C overnight and centrifuged at 1000×g for 20min. The serum was collected and portions were

dispensed into 1ml aliquots and stored in liquid nitrogen until use (Kelly & Nielsen 1985).

Virus

IPNV isolate Jasper used in most studies was serotype A_9, subtype Jasper. This virus was isolated from diseased brook trout (*Salvelinus fontinalis*) in Maligne River Hatchery, Alberta, Canada (Yamamoto 1974) and was obtained from Dr. B. Nicholson, University of Maine, Orono, ME, U.S.A. and originally donated by Barry Hill (DAFF, Waymouth, U.K.). The virus was passaged for years in our laboratory. The other IPNV isolate used was serotype A_2, subtype Sp, Thailand. This isolate was isolated from diseased snakehead fish (*Ophicephalus striatus*) in Thailand (Wattanavijarn et al 1988) and was obtained from Wattanavijarn.

Serum inhibition of virus *in vitro*

The RTG-2 cell line (Wolf and Quimby 1962) and other cell lines used for each experiment were propagated in 24 well microtitre plates as described by Caswell-Reno et al (1989). Three replicate wells were prepared for each condition. In this experiment, three types of media were used: Eagle's Minimum Essential Medium (MEM) without serum=MEM-0; MEM+10% fetal bovine serum=MEM-10; MEM+10% fetal bovine

serum+1%RTS=MEM-RTS. Virus was diluted with MEM-10 or MEM-RTS to give the final virus concentration with $10^4 TCID_{50}$/ml. Three hundred μL of diluted virus were added onto a confluent monolayer in each well and incubated at room temperature for two hours. After the incubation time, the inoculated monolayers were washed three times with MEM-0 and then 1ml of either MEM-10 or MEM-RTS was added. Uninoculated controls were treated with either medium, MEM-10 or MEM-RTS, as appropriate. Cells were incubated at 18°C for seven days in an incubator in which 5% CO_2 was supplied. The level of cytopathogenic effect (CPE) was monitored daily and scaled from 0 (no CPE) to 4 (complete CPE) ordinal scale. On the seventh day after exposure, cell culture supernatant from each well was harvested, pooled, and held in liquid nitrogen until they were titrated.

Virus titration

The endpoint dilution method as described in Caswell-Reno et al. (1986) was used for virus titration. Virus samples were serially diluted 10-fold with MEM-0 and then 100μL of each of diluted virus were added to each of four wells of a 96 well plate containing monolayers of CHSE-214 cells (Lannan et al. 1984). After incubation at 18°C for 7 days, wells showing CPE were counted to determine 50% tissue culture infectious dose ($TCID_{50}$/ml) (Spearman 1908).

Effect of RTS pretreatment of cells

A modification of the method described by de Sena and Rio (1975) was employed to determine inhibition induction by RTS on RTG-2 cells. Rainbow trout serum (RTS) was diluted in MEM-10 at the ratios 5.0%, 2.5%, 1.8%, and 1.0%. Each diluted serum sample was incubated on three replicates wells in 24 well microtitre plates containing confluent RTG-2 cells (approximately 3×10^5 cells/well). Control wells were pretreated with MEM-10. After 24 hours of preincubation, the wells were washed three times with MEM-0 and infected with 300µL of IPNV-Jasper at a concentration of 10^4 $TCID_{50}$/ml. After two hours of incubation time the inoculated monolayers were washed three times with MEM-0 and then 1 ml of MEM-10 was added to each. After 7 days incubation, virus titer was determined as described above.

MEM-RTS was preincubated on three replicates wells in 24 well microtiter plates prepared with RTG-2 cells. Control wells were pretreated with MEM-10. After two hours of preincubation, cells were washed 0, 1, 3, 5, 10 times each with MEM-0. Then cells were exposed to 300µL of IPNV-Jasper containing 10^4 $TCID_{50}$/ml for 2 hours and then washed three times with MEM-0. All cells were then incubated with MEM-10 for 7 days and then they were titrated as above.

Effect of time of serum addition on inhibition

RTG-2 cells were grown with MEM-10 in 24 well microtiter plates (approximately 3×10^5 cells/well) and exposed to 300 µL of 10^4 $TCID_{50}$/ml of IPNV-Jasper in either MEM-10 or MEM-RTS for 2 h at 18°C as described above. Three replicates of 12 different conditions in RTS addition were employed in the experiments (Figure 4.1). As shown in Figure 4.1, virus was exposed to MEM-RTS or MEM-10 before cell infection at different time intervals (A), while MEM-RTS or MEM-10 was added to cells after viral infection at different time intervals (B). At the time 0, virus and MEM-RTS or MEM-10 were added to cells concurrently. After two hours of incubation, all wells were washed three times with MEM-0 and then wells were incubated with MEM-10 or MEM-RTS as appropriate. In the time B groups, cells were incubated with MEM-10 until the time of RTS addition. On the 7th day, virus titer was determined from each well of MEM-RTS or MEM-10 at the specific time of MEM-RTS addition.

Effect of 2h pretreatment with RTS only vs 7 day incubation with RTS

Five hundred µL of Thailand IPNV isolate at a concentration of $10^9$$TCID_{50}$/ml were mixed with either 4.5 ml of MEM-RTS or 4.5 ml of MEM-10 as a control for two hours. The virus incubated in MEM-RTS was diluted with MEM-RTS or with MEM-10 to a virus concentration of 10^9 to $10^0$$TCID_{50}$/ml while the virus incubated in MEM-10 was diluted with MEM-10 to a concentration of 10^9 to $10^0$$TCID_{50}$/ml. Since the end point

of RTS inhibition in a preliminary experiment was RTS 1:800 dilution, a further 10^{-1} dilution with MEM-10 of virus (which was originally incubated in 1:100 RTS) produced a concentration of less than the lowest inhibitory concentration of RTS. Thus, if virus was affected by RTS, the effect was caused by the initial two hours pretreatment rather than residual RTS in the incubation medium. One hundred μL of each viral concentration ranging from $10^{0}TCID_{50}$/ml to $10^{8}TCID_{50}$/ml from each condition were added to each well of 96 well plates prepared with CHSE-214 cell monolayer. After incubation at 18°C for 7 days, 50% tissue culture infectious dose ($TCID_{50}$/ml) was compared with each dilution plates of three types of viral conditions.

RTS and CHS inhibition on RTG-2 and CHSE-214 cells

In order to determine if homologous cell line and serum pairings affected inhibitory activity, both chinook salmon and rainbow trout were tested. Two cell lines, RTG-2 and CHSE-214, were propagated in 24 well microtiter plates as described above. IPNV-Jasper was diluted with MEM-10 (control) or MEM-RTS or MEM+10% fetal bovine serum+1% Chinook salmon serum (MEM-CHS) to give the final virus concentration of $10^{4}TCID_{50}$/ml. Three hundred μL of each diluted virus in MEM-10, MEM-RTS, or MEM-CHS were added into both RTG-2 and CHSE-214 cells. After two hours of incubation, the inoculated monolayers were washed three times with MEM-0 and then 1ml of either medium MEM-10, MEM-RTS, or MEM-CHS was added as appropriate. Cells were incubated for seven days and then virus titer was determined.

Sensitivity to RTS following passage in RTG-2 or CHSE-214 cells

In order to determine if passage in homologous or heterologous cells affected inhibitory activity, IPNV-Thailand was passaged five times in CHSE-214 or RTG-2 cells. RTS sensitivity was compared from passage one and five in either CHSE-214 or RTG-2. To check for alteration of RTS sensitivity, viral inactivation after two hours of incubation with MEM-RTS was determined. One hundred μL of passage one and passage five virus at a concentration of $10^8 TCID_{50}$/ml originated from either CHSE-214 or RTG-2 were incubated with 900μL of MEM-RTS or MEM-10 for 2 hours. Each of these passages from the two cell lines was serially diluted with MEM-10 to a concentration of $10^0 TCID_{50}$/ml. One hundred μL of each viral dilution from MEM-RTS and MEM-10 origin were inoculated onto 96 replicate wells of a microtiter plate containing CHSE-214 cells instead of RTG-2 cells. Seven days later, virus titer was compared between MEM-RTS or MEM-10.

Effect of cell density on RTS inhibition

RTG-2 cells in $75cm^2$ flask were trypsinized and then serially diluted in 2-fold steps to 2^{-5} with MEM-10. Diluted cells were seeded into 24 well microtiter plates (four replicates for each cell concentration). Four days later, cells were counted in a hemo-cytometer from one of four wells of each cell dilution after trypsinization. After determination of cell concentration, virus inhibition test from the those other replicate

wells were conducted with IPNV-Jasper. The test methods were the same as serum inhibition tests *in vitro*.

RTS inhibition in salmonid and non-salmonid cell lines

Thirteen continuous teleost cell lines were tested for their ability to support the inhibition of IPNV by RTS. Cells included those from marine and freshwater fishes as well as salmonid and non-salmonid fishes. Both epithelial and fibroblastoid cell types were represented. Each cell line was propagated in 24 well microtiter plates. Three replicate wells were tested for each cell line when cell lines were 100% confluent. Detailed information about the cell lines used is listed in Table 4.1. The inhibition test for RTS was the same as described earlier with RTG-2 cells.

Species range of serum inhibitory activity against IPNV

Sera were collected from seven teleost fishes to determine their ability to inhibit IPNV. Two isolates known to be highly sensitive to inhibition by RTS were selected for use: Thailand (A_2, Sp serotype) and Jasper (A_9, Jasper serotype). Sera were collected from the species listed in the Table 4.2 prepared as for RTS, and held in liquid nitrogen until used in these experiments. All sera were diluted in MEM-10 to 1:100. The inhibition test was carried out on RTG-2 cells as described above

Figure 4.1 Scheme for testing the effect of RTS addition time on the serum inhibition to IPNV-Jasper. Medium with rainbow trout serum (MEM-RTS) or without RTS (MEM-10) (control) was added to virus and/or cells at 12 different times. IPNV-Jasper was exposed to MEM-RTS (V+RTS), or MEM-10 (V-RTS) prior to cell infection at different times (A); conversely, MEM-RTS or MEM-10 was added to cells after viral infection at different times (B). At time 0, virus and MEM-RTS or MEM-10 was added to cells concurrently. On the 7^{th} day after infection of the cells with virus, virus titer was compared from MEM-RTS or MEM-10 at each time interval.

	A				B						
Case#	-2h	-1h	-0.5h	0 (infec.)	10min	30min	1h	2h (wash)	8h	16h	24h	48h
1	V±RTS			V±RTS				wash±RTS				
2		V±RTS		V±RTS				wash±RTS				
3			V±RTS	V±RTS				wash±RTS				
4				V±RTS				wash±RTS				
5				V+MEM	±RTS			wash±RTS				
6				V+MEM		±RTS		wash±RTS				
7				V+MEM			±RTS	wash±RTS				
8				V+MEM				wash±RTS				
9				V+MEM				wash+MEM	±RTS			
10				V+MEM				wash+MEM		±RTS		
11				V+MEM				wash+MEM			±RTS	
12				V+MEM				wash+MEM				±RTS

Figure 4.1

Table 4.1 Characteristics of 13 continuous teleost cell lines (9 salmonid cell lines and 4 non-salmonid cell lines) tested for their ability to support the virus inhibition of RTS inhibitor.

Cell Line Abbreviaion	Tissue Source	Species of Origin	Cell Morphology[a]	Reference
CHH-1	Chum Heart-1	*Oncorhynchus keta*	E	Lannan et al. (1984)
CHSE-114	Chinook Salmon Embryo	*Oncorhynchus tshawytscha*	E	Lannan et al. (1984)
CHSE-214	Chinook Salmon Embryo	*Oncorhynchus tshawytscha*	E	Lannan et al. (1984)
KO-6	Kokanee Ovary	*Oncorhynchus nerka*	E	Lannan et al. (1984)
RTG-2	Rainbow Trout Gonad	*Oncorhynchus mykiss*	F	Wolf & Quimby (1962)
RTH-149	Rainbow Trout Hepatoma	*Oncorhynchus mykiss*	E	Lannan et al. (1984)
SSE-5	Sockeye Salmon Embryo	*Oncorhynchus nerka*	E	Lannan et al. (1984)
STE-137	Steelhead Trout Embryo	*Oncorhynchus nerka*	E	Lannan et al. (1984)
YNK	Yamame Kidney	*Oncorhynchus masou*	F	Watanabe et al. (1978)
BB	Brown Bullhead	*Ictalurus nebulosus*	E	Wolf & Quimby (1969)
BF-2	Bluegill Sunfish	*Lepomis machrochirus*	F	Wolf & Quimby (1966, 1969)
CCO	Channel Catfish Ovary	*Ictalurus punctatus*	F	Bowser (1976)
PHE-184	Pacific Herring Embryo	*Clupea harengus pallasi*	E	Lannan & Olson unpublished

a: epithelioid(E); fibroblastic(F)

Table 4.2 Sources and characteristics of seven fish sera utilized in the study of inhibition of IPNV.

Serum Designation	Species	W[a]/H	Weight[b]	N[c]
RTS (rainbow trout serum)	*Oncorhynchus mykiss*	H	600 g	30
COS (coho salmon serum)	*Oncorhynchus kisutch*	H	2 kg	10
CHS (chinook salmon serum)	*Oncorhynchus tshawytscha*	H	2 kg	10
BTS (brook trout serum)	*Salvelinus fontinalis*	H	500 g	30
FLS (starry flounder serum)	*Platichthys stellatus*	W	2 kg	1
SAS (sablefish serum)	*Anoplopoma fimbria*	W	5 kg	1
HES (pacific herring serum)	*Clupea harengus pallasi*	W	100 g	30

a: fish obtained from wild (W) or hatchery (H)

b: mean weight of fish from which serum was obtained

c: number of individuals used for serum pool

RESULTS

To determine the extent of the inhibitory capacity of normal rainbow trout serum (RTS) against IPNV, a dilution series was tested against 3 isolates of virus. Two isolates West Buxton and Buhl (both serotype A_1) were inhibited by at least $1\log_{10}TCID_{50}$/ml at dilutions of 1:300 and 1:1000, respectively. The Jasper isolate (serotype A_9) was inhibited at a dilution of 1:1000. Thus, for all of the experiments performed here, a 1:100 dilution of serum was used.

Effect of RTS pretreatment of cells

To determine if RTS induces inhibition during a 24h pretreatment of RTG-2 cells, as does interferon, unexposed cells were pretreated with MEM-RTS at various concentrations 24h prior to infection with virus, followed by thorough washing of the cells. As shown in Figure 4.2, no significant difference in virus titer was noted between cells pretreated with MEM-RTS and cells pretreated with MEM-10. Virus titer in all cells pretreated with MEM-RTS ranged from $10^{6.6}TCID_{50}$/ml to $10^{7.0}TCID_{50}$/ml. This range of virus titer was not statistically different from the titer of cells treated with MEM-10 ($10^{6.5}TCID_{50}$/ml) ($F_{(4,10)}=1.167$, $p=0.3818$).

To determine if RTS inhibition was caused by the attachment of some serum component(s) to a cell receptor for the virus (thereby masking a viral receptor), cells were pretreated prior to viral infection. As shown in Figure 4.3, there was approximately a 10-

fold reduction in virus titer after a pretreatment of cells with RTS followed by pretreatment. However the inhibition levels from pretreated cells, approximately 10^{-1} $TCID_{50}$/ml reduction, were not significantly different from control MEM-10 ($F_{(5,12)}$=1.156, p=0.3846). No difference in inhibition level was obtained by different number of washes. The inhibition levels from cells pretreated with MEM-RTS were not as high as the control-RTS inhibition, 10^{-5} $TCID_{50}$/ml reduction, ($F_{(5,12)}$=4.847, p=0.0117).

Effect of time of serum addition on inhibition

To determine the effect of time of RTS addition to virus and cells on virus inhibition, MEM-RTS was mixed with virus at different times before viral infection, or to host cells at different times after viral infection. The results indicated MEM-RTS inhibition level was dependent on the time at which virus was exposed to MEM-RTS (Figure 4.4). The strongest inhibition ($10^{-6.3}$ $TCID_{50}$/ml reduction in virus titer) was obtained when the virus was exposed to MEM-RTS two hours before infection of cells. The inhibition level, however, was significantly decreased by 1 hour pre-exposure to a $10^{-2.5}$ $TCID_{50}$/ml reduction in virus titer, a level which was maintained for 16 h postinfection and was statistically significant ($p<0.05$). In other conditions of MEM-RTS addition, no significant inhibition occurred at 24 or 48 hours postinfection (df=1, F=6.721, p=0.0605).

Figure 4.2 The effect of RTS on inhibition of IPNV-Jasper after pretreatment of RTG-2 cells 24 hours before infection. Each of diluted RTS sera (MEM-RTS) was pre-incubated with cells for 24 hours before viral infection then washed from the cell monolayer just prior to exposure to virus. Virus titers in RTS pretreatment were compared to the control, MEM (=MEM-10), seven days postexposure. Inhibition above $10^{1.0}$ is considered significant.

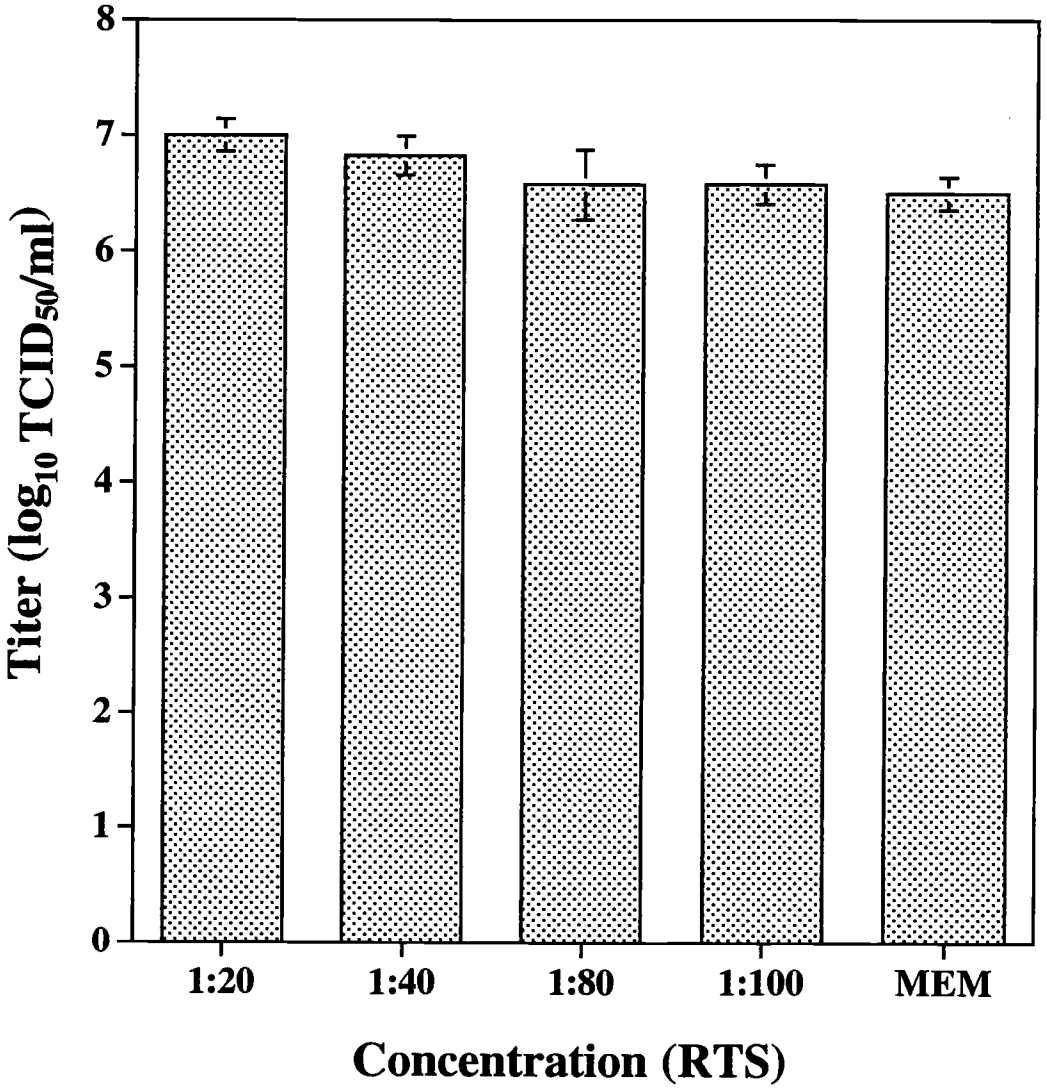
8
7
6
5
4
3
2
1
0
Titer (log_{10} $TCID_{50}$/ml)
1:20
1:40
1:80
1:100
MEM
Concentration (RTS)

Figure 4.2

Figure 4.3 The effect of preincubation of RTG-2 cells with RTS (=MEM-RTS) before viral infection and the effect of washing on inhibitory activity of RTS against IPNV-Jasper. RTG-2 cells was preincubated with RTS for 2 hours before viral infection. Controls, MEM or RTS, were preincubated with MEM (=MEM-10). Treated cells were washed from 0 times to 10 times before viral infection; controls were not washed. Inhibition of virus titer was measured seven days postexposure. Inhibition above $10^{1.0}$ is considered significant.

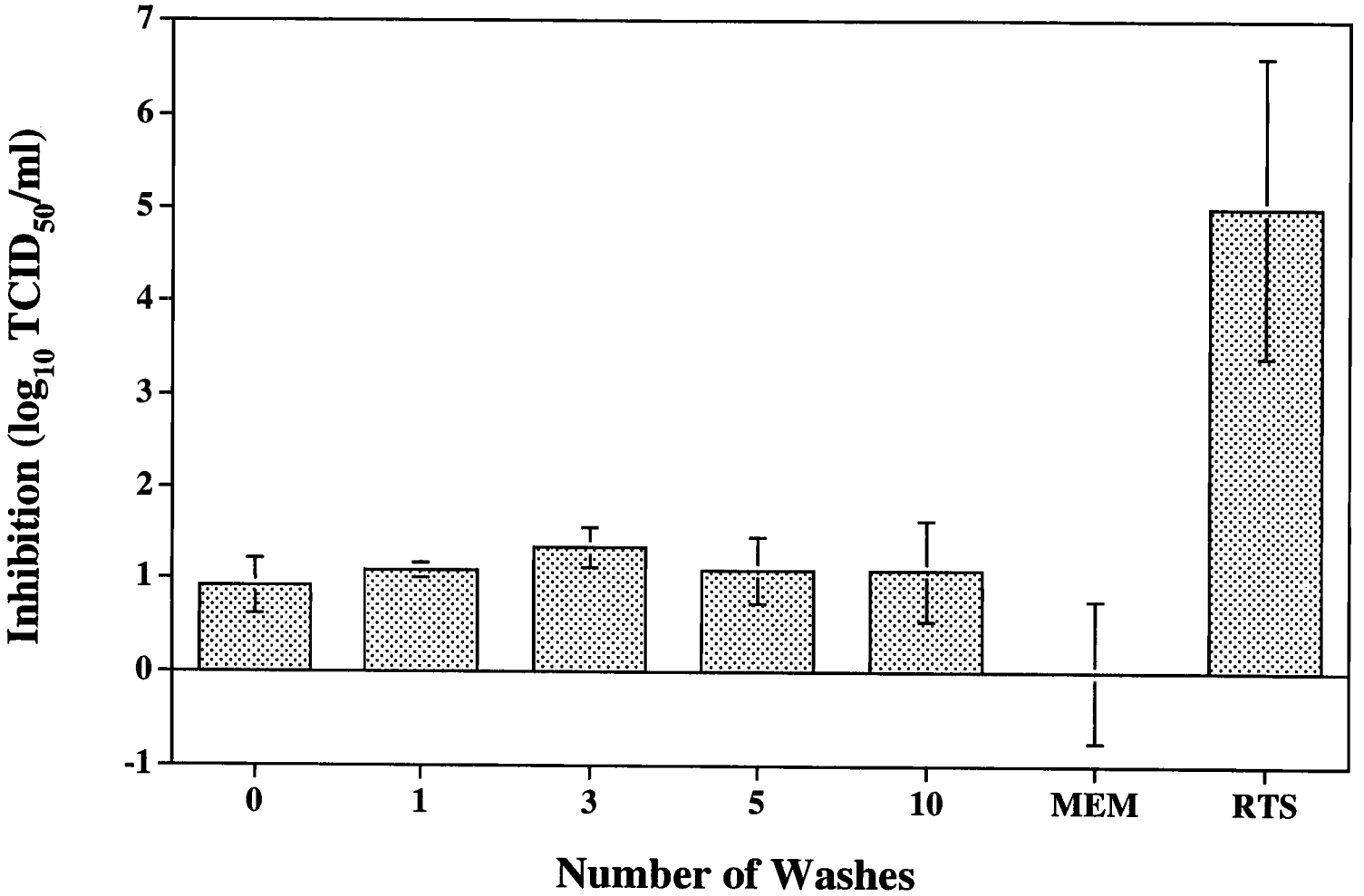
Inhibition (log_{10} $TCID_{50}$/ml)
7
6
5
4
3
2
1
0
-1
0
1
3
5
10
MEM
RTS
Number of Washes

Figure 4.3

Figure 4.4 Time defendence of RTS inhibitory activity. Medium with rainbow trout serum (MEM-RTS) was added to IPNV-Jasper prior to exposure of cells (negative times), concurrently with (t=0), and at various times after addition of virus to RTG-2 cells. Inhibitory activity of RTS was measured at seven day postexposure. Inhibition above $10^{1.0}$ is considered significant.

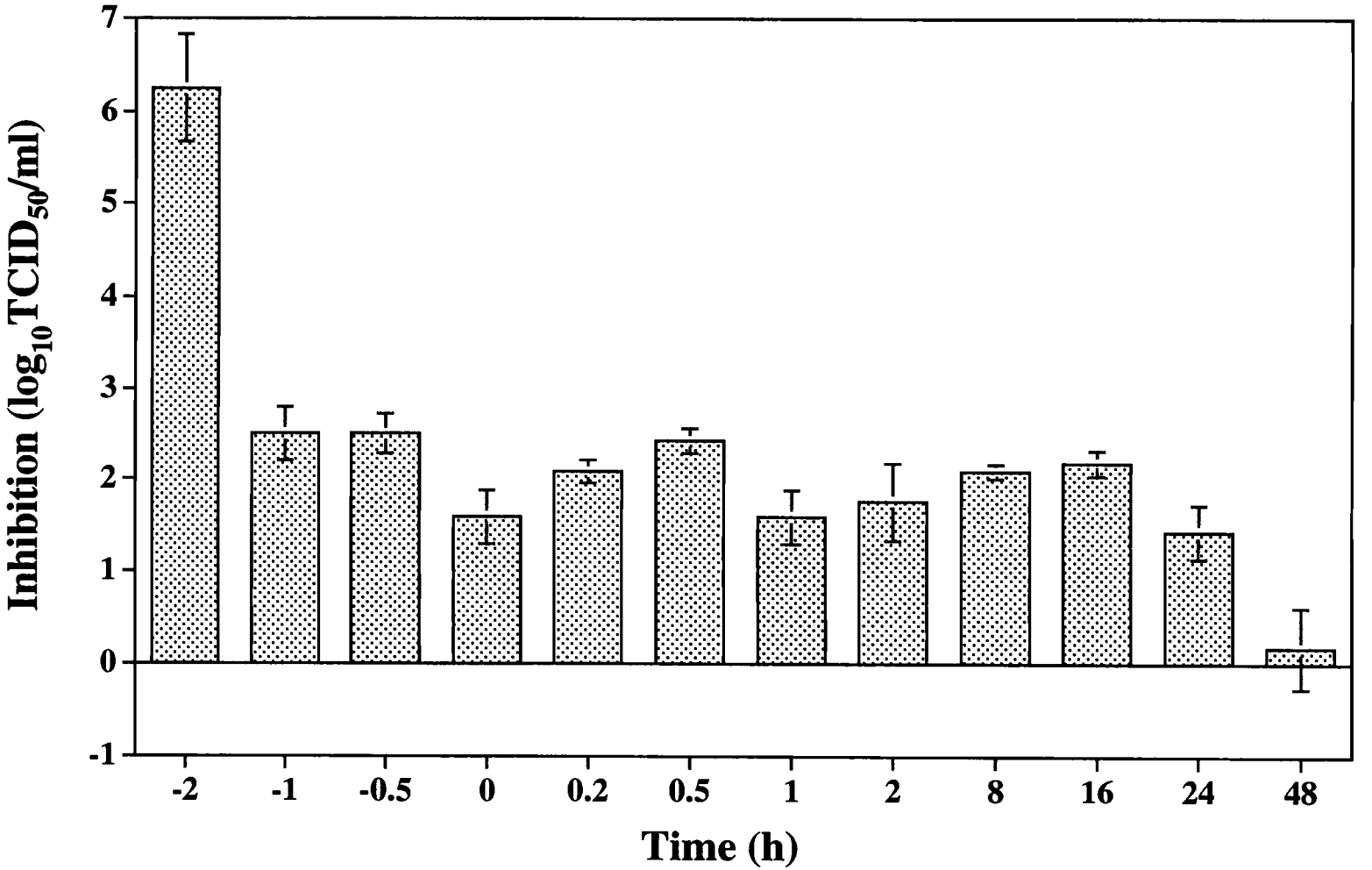
Inhibition ($log_{10}TCID_{50}$/ml)
7
6
5
4
3
2
1
0
-1
-2
-1
-0.5
0
0.2
0.5
1
2
8
16
24
48
Time (h)

Figure 4.4

Effect of 2h pretreatment with RTS only vs 7 day incubation with RTS

To determine if RTS inhibitor has a direct effect on IPNV, such as agglutination, virus titer was determined after incubation with MEM-RTS. Virus titer with MEM-10 only was $10^{8}TCID_{50}$/ml. Treatment of virus for 2 h with MEM-RTS, followed by dilution and incubation with MEM-10 for 7 day, reduced the titer to $10^{-4.5}TCID_{50}$/ml reduction. If MEM-RTS pretreatment was combined with 7 day incubation in the presence of MEM-RTS, the titer was further reduced to $10^{-7.0}TCID_{50}$/ml reduction. This result suggests that virus is directly inhibited by RTS and more strongly inhibited when RTS is present during viral replication.

RTS and CHS inhibition on RTG-2 and CHSE-214 cells

In order to determine if serum inhibition against IPNV is related to serum source or to the species of host cell, we determined cross activity of inhibition in two cell lines, RTG-2 and CHSE-214 cells, using sera from two sources, rainbow trout serum (RTS) and Chinook salmon serum (CHS). virus inhibition was cell line dependent (Figure 4.5). IPNV was more efficiently inhibited, $10^{-3.0}TCID_{50}$/ml reduction, by MEM-RTS in RTG-2 cell line. MEM-RTS completely protected the RTG-2 cell line; however, no inhibition occurred in CHSE-214 cells ($df=1$, $F=0.053$, $p=0.8298$). On the other hand, MEM-CHS did not show high inhibition such as that seen with MEM-RTS. Although MEM-CHS inhibited viral replication more effectively in CHSE-214 cells, $10^{-0.8}TCID_{50}$/ml reduction,

than that in RTG-2 cells, $10^{-0.2}TCID_{50}$/ml reduction, the inhibition level not significant ($10^{-1}TCID_{50}$/ml reduction). However, the inhibition level ($10^{-0.8}TCID_{50}$/ml reduction) was statistically different than control (df=1, F=14.286, p=0.0194).

In terms of CPE level, until the fourth day, MEM-RTS and MEM-CHS showed a similar level of CPE (level 0) in RTG-2 cells while CPE (level 1) in positive control (MEM-10) was shown in 2 days after viral infection (data not shown). However after the fourth day, CPE level in MEM-CHS was rapidly increased to the same level of CPE (level 4) and virus titer of positive control (MEM-10) (Figure 4.5).

Sensitivity to RTS following passages in RTG-2 and CHSE-214 cells

We determined the change of RTS sensitivity of virus in order to determine if RTS sensitivity is related to host cells in which virus is replicated. We observed that RTS sensitivity of virus can be changed by the cell line in which virus is produced (Figure 4.6). The IPNV isolate Thailand passaged once in CHSE-214 or RTG-2 showed similar RTS inhibition levels, approximately $10^{-3.4}$/ml TCID50 reduction (df=1, F=0.060, p=0.8147). Five viral passages in CHSE-214, however, affected RTS sensitivity, resulting in only $10^{-1.9}TCID_{50}$/ml reduction (df=1, F=13.500, p=0.0104). On the other hand, IPNV isolate Thailand passaged in RTG-2 showed only $10^{0.4}$ $TCID_{50}$/ml difference between passage one and passage five (df=1, F=0.960, p=0.3650): $10^{-3.3}$ $TCID_{50}$/ml reduction in passage one and $10^{-2.9}TCID_{50}$/ml reduction in passage five. This result indicates that RTS sensitivity of IPNV is related to host cells in which IPNV is prepared.

Effect of cell density on RTS inhibition

RTS inhibition was tested at different densities of RTG-2 cells in order to determine if RTS inhibition is dependent on cell density in which RTS inhibition is tested. RTS inhibition was highly dependent on cell density (Figure 4.7). The confluency levels of cell monolayer at viral infection were varied: approximately 70% confluency ($1x10^5$ cells/ml); 80% ($1.2x10^5$ cells/ml); loosely 100% ($2x10^5$ cells/ml); tightly 100% confluent ($3x10^5$ cells/ml and $8x10^5$ cells/ml). The inhibition level ranged from no significant inhibition at the cell density of $\leq 2x10^5$/ml to $10^{-6.5}TCID_{50}$/ml reduction in virus titer at a cell density of $8x10^5$/ml. Until the cell density reached $2x10^5$/ml, inhibition level was insignificant with maximum inhibition of $10^{-1.1}TCID_{50}$/ml reduction. Even though the inhibition at $1.2x10^5$ cells/ml was $10^{-1.1}TCID_{50}$/ml reduction, it was not statistically significant from the control ($df=1$, $F=3.425$, $p=0.1379$). However, above a cell density of $3x10^5$, the inhibition level was very high and statistically significant; $10^{-6.3}$ $TCID_{50}$/ml reduction ($df=1$, $F=1569.565$, $p=<0.0001$) at $3x10^5$ cells/ml and $10^{-6.5}$ $TCID_{50}$/ml reduction ($df=1$, $F=150.544$, $p=0.0003$) at $8x10^5$ cells/ml. At the latter two cell densities, cell monolayers were 100% confluent.

Figure 4.5 The comparison of serum inhibitory activity of chinook salmon serum (CHS) and RTS, against IPNV-Jasper, on two cell lines, RTG-2 and CHSE-214. The virus was grown on RTG-2 or CHSE-214 cells. Each virus in MEM-10 (control), MEM-CHS, or MEM-RTS was added into both RTG-2 and CHSE-214 cells. After two hours, cells were washed and then either medium MEM-10, MEM-RTS, or MEM-CHS was added as appropriate. Inhibition of virus titer was measured seven days postexposure. Inhibition above $10^{1.0}$ is considered significant.

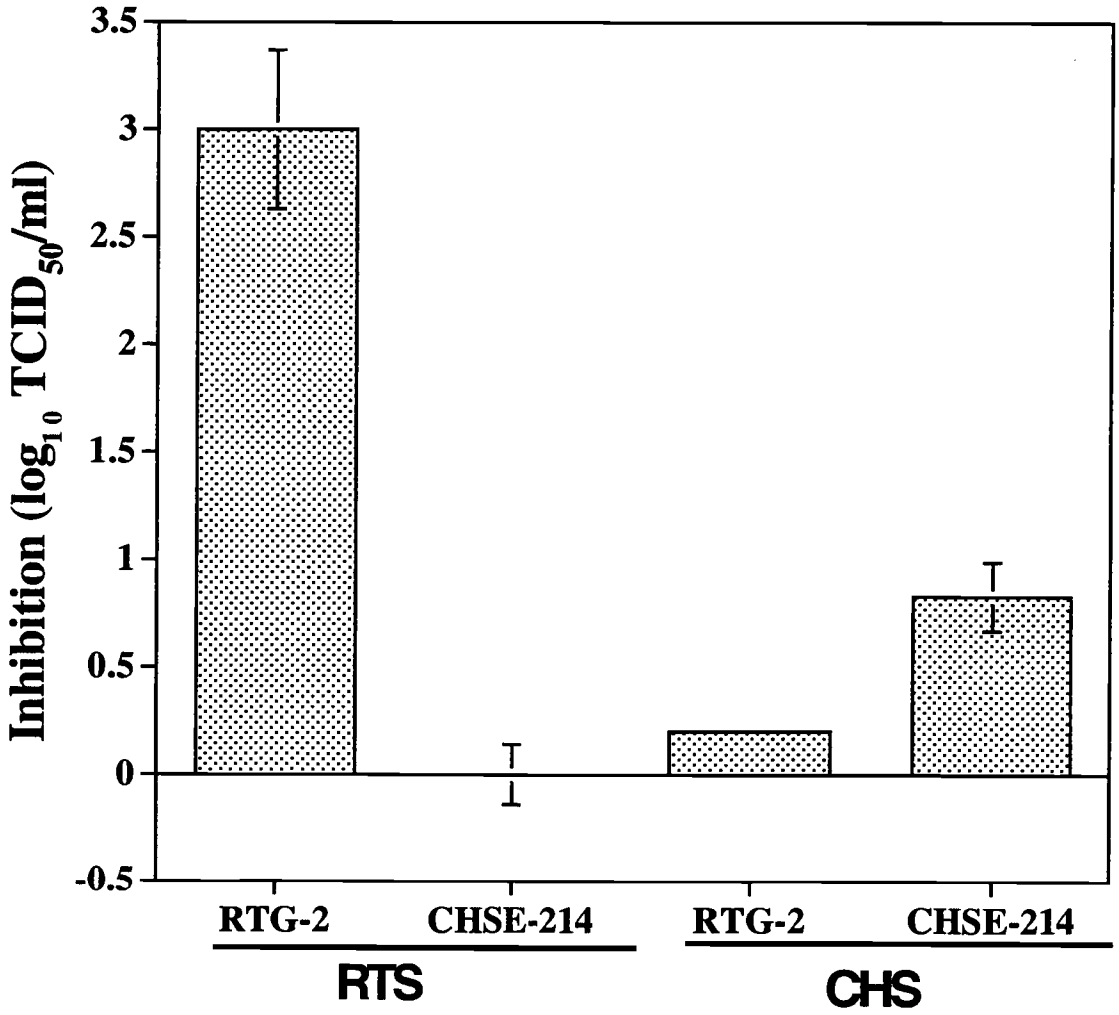
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Inhibition (log10 TCID50/ml)
RTG-2
CHSE-214
RTG-2
CHSE-214
RTS
CHS

Figure 4.5

Figure 4.6 The change of RTS sensitivity after passaging IPNV-Thailand in two different cell lines, RTG-2 or CHSE-214. IPNV-Thailand was passaged five times in CHSE-214 or RTG-2 cells. RTS sensitivity was compared from passage one (P1) and passage 5 (P5) in either CHSE (=CHSE-214) or RTG (=RTG-2). After two hours of incubation with MEM-10 or MEM-RTS, inactivation of each passaged virus was measured to determine the change of RTS inhibition. Inhibition above $10^{1.0}$ is considered significant.

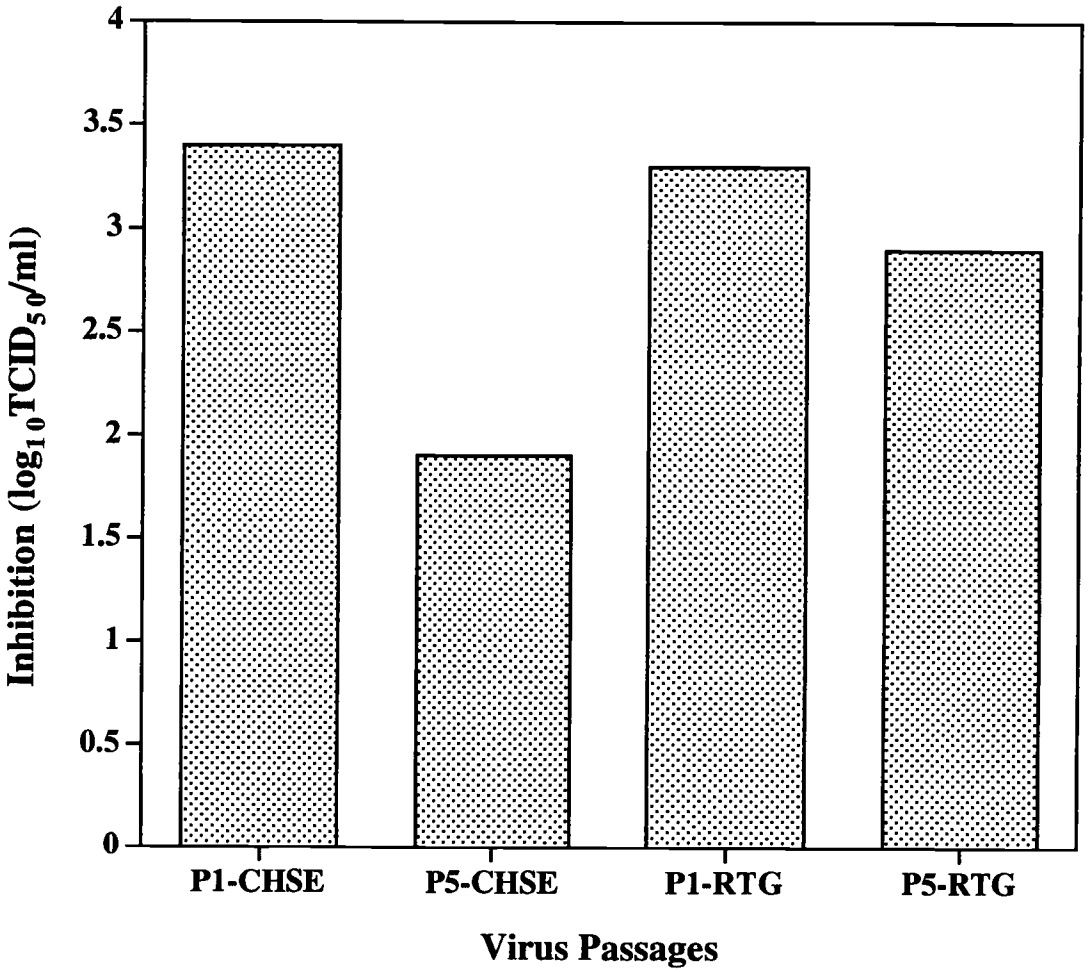
4
3.5
3
2.5
2
1.5
1
0.5
0
Inhibition ($log_{10}TCID_{50}$/ml)
P1-CHSE
P5-CHSE
P1-RTG
P5-RTG
Virus Passages

Figure 4.6

Figure 4.7 The effect of cell density on RTS inhibitory activity. The confluency (%) of cell monolayer (RTG-2 cells) at each cell density was as follows: 70% ($1x10^5$/ml), 80% ($1.2x10^5$/ml), loosely 100% ($2x10^5$/ml), tightly 100% ($3x10^5$/ml and $8x10^5$/ml). Virus inhibition test at each cell densities was conducted with IPNV-Jsaper. Inhibition of virus titer was measured seven days postexposure. Inhibition above $10^{1.0}$ is considered significant.

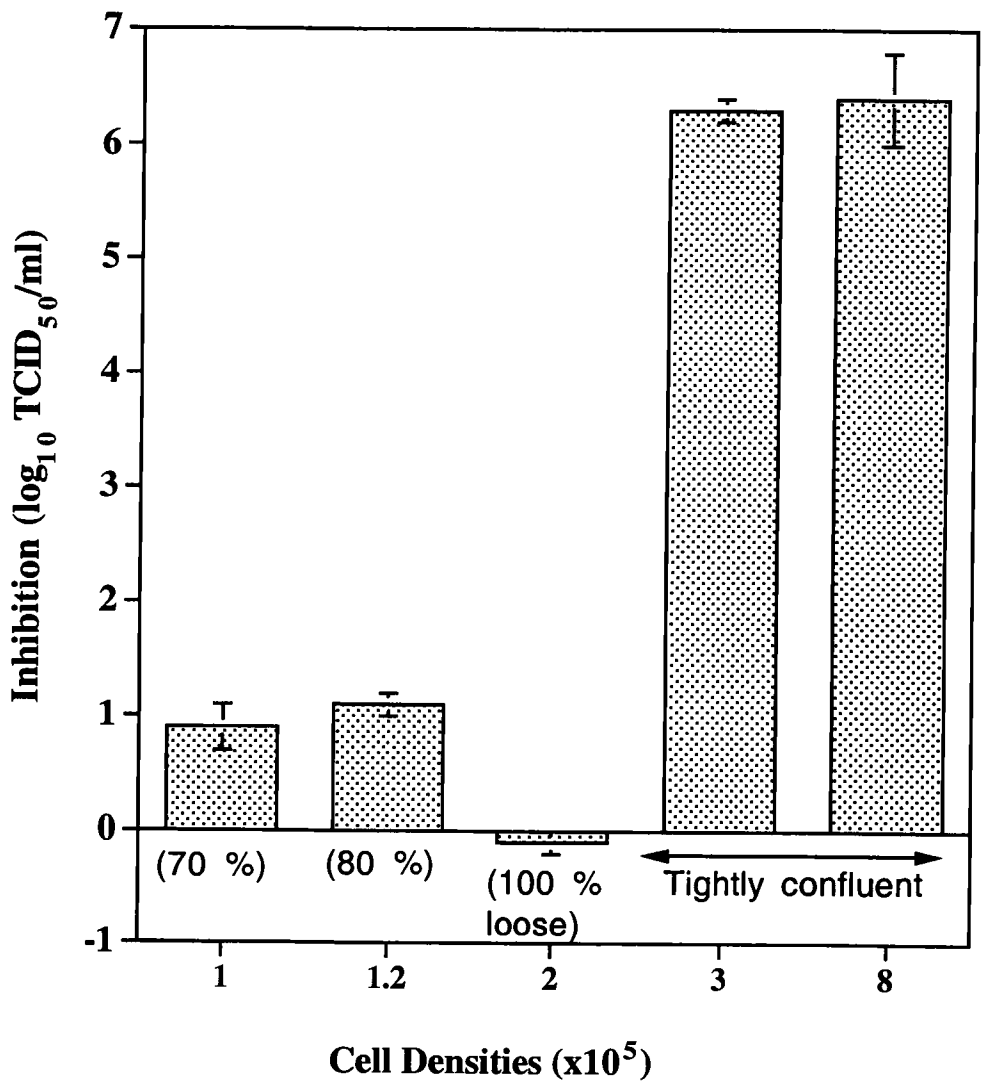
7
6
5
4
3
2
1
0
-1
Inhibition (log_{10} $TCID_{50}$/ml)
(70 %)
(80 %)
(100 % loose)
Tightly confluent
1
1.2
2
3
8
Cell Densities (x10^5)

Figure 4.7

Virus inhibition in salmonid and non-salmonid cell lines

Nine salmonid and four non-salmonid cell lines were tested to determine if virus inhibition of RTS inhibitor has cell line specific. It was found that serum inhibition had host-cell specificity; RTS (salmonid serum) showed higher inhibition in salmonid cell lines than in non-salmonid cell lines and showed the highest inhibition in host cells from the homologous species (RTG-2 cells, RTH-149) among salmonid cell lines. In nine salmonid cell lines (Figure 4.8), strong RTS inhibition was found in 5 cell lines, YNK ($10^{-6.0}$TCID$_{50}$/ml reduction), CHH-1 ($10^{-6.0}$TCID$_{50}$/ml reduction), STE-137 ($10^{-6.1}$TCID$_{50}$/ml reduction), RTG-2 ($10^{-6.6}$TCID$_{50}$/ml reduction) and RTH-149 ($10^{-6.6}$TCID$_{50}$/ml reduction); however, inhibition was not found in 4 cell lines ($<10^{-0.1}$TCID$_{50}$/ml reduction), KO-6, SSE-5, CHSE-214 and CHSE-114. Host cell lines, RTG-2 and RTH-149, showed the highest virus inhibition by RTS. In four non-salmonid cell lines (Figure 4.9), two (BF-2 and PHE-184) did not show significant RTS inhibition ($<10^{-1.0}$ TCID$_{50}$/ml reduction); two ictalurid cell lines, CCO and BB, showed significant virus inhibition; $10^{-1.9}$TCID$_{50}$/ml (df=1, F=264.5, p=<0.0001) and $10^{-3.8}$TCID$_{50}$/ml reduction (df=1, F=184.091, p=0.0002) respectively. However, these two non-salmonid cell lines demonstrating strong inhibition showed much less inhibition by RTS compared to inhibition in five salmonid cell lines showing significant inhibition (df=1, F=40.664, p=0.0014). Virus inhibition was not dependent on cell morphology, epithelioid or fibroblastic.

Figure 4.8 Inhibition of IPNV-Jasper by RTS in salmonid cell lines. Virus inhibition was measured seven days postexposure in nine salmonid cell lines: RTG-2 (Rainbow Trout Gonad-2); RTH-149 (Rainbow Trout Heart-149); STE-137 (Steelhead Salmon Embryo-137); YNK (Yamame Kidney); CHH-1 (Chum Heart-1); KO-6 (Kokanee Ovary-6); SSE-5 (Sockeye Salmon Embryo-5); CHSE-214 (Chinook Salmon Embryo-214); CHSE-114 (Chinook Salmon Embryo-114). Inhibition above $10^{1.0}$ is considered significant.

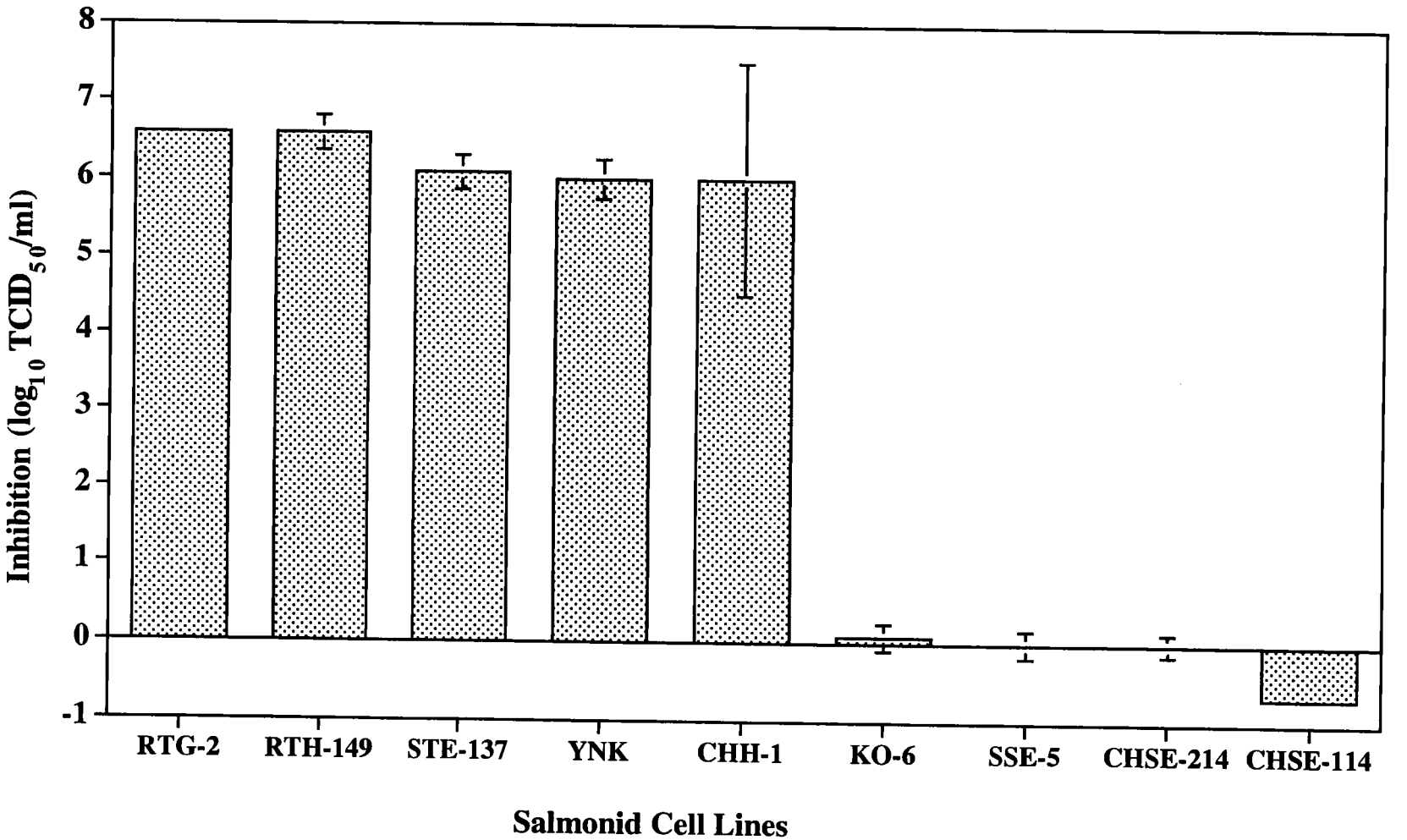
8
7
6
5
4
3
2
1
0
-1
Inhibition (log_{10} $TCID_{50}$/ml)
RTG-2
RTH-149
STE-137
YNK
CHH-1
KO-6
SSE-5
CHSE-214
CHSE-114
Salmonid Cell Lines

Figure 4.8

Figure 4.9 Inhibition activity against IPNV-Jasper in non-salmonid cell lines. Virus inhibition of RTS inhibitor was measured seven days postexposure in four non-salmonid cell lines: CCO (Channel Catfish Ovary); BB (Brown Bullhead); BF-2 (Bluegill Sunfish-2); PHE-184 (Pacific Herring Embryo-184). Inhibition above $10^{1.0}$ is considered significant.

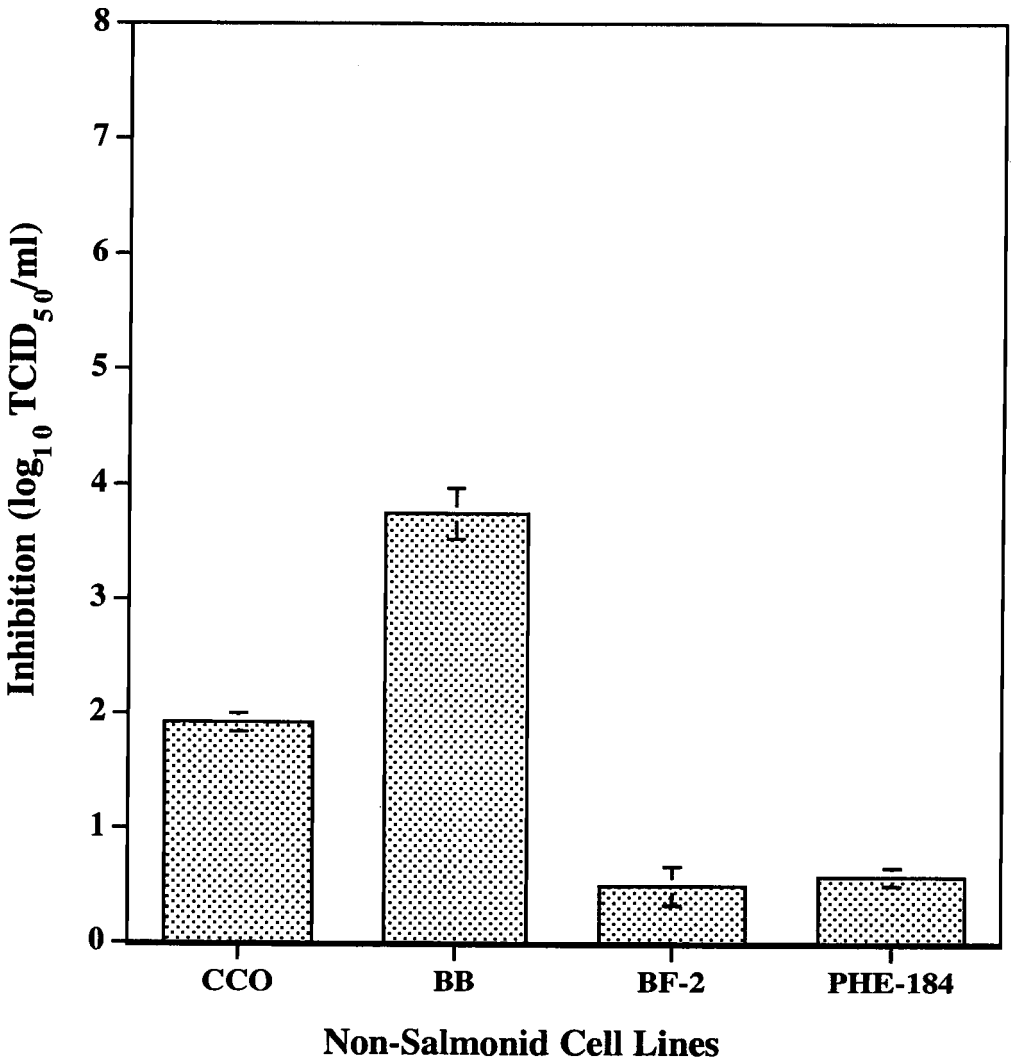
8
7
6
5
4
3
2
1
0
Inhibition (log_{10} $TCID_{50}$/ml)
CCO
BB
BF-2
PHE-184
Non-Salmonid Cell Lines

Figure 4.9

Species range of serum inhibitory activity against IPNV

The inhibition activity in sera of seven species of fish was tested to determine the species range of virus inhibition against two isolates known to be sensitive to RTS. Inhibition activity showed high variation depending on serum sources and virus tested (Figure 4.10). No significant serum inhibition was found in three non-salmonid sera, FLS, SAS, and HES: FLS ($10^{-0.6}$TCID$_{50}$/ml reduction), SAS ($10^{0.4}$TCID$_{50}$/ml increase), HES ($10^{-0.1}$TCID$_{50}$/ml reduction) in IPNV-Jasper; FLS ($10^{0.3}$TCID$_{50}$/ml increase), SAS ($10^{0.5}$TCID$_{50}$/ml increase), HES ($10^{0.6}$TCID$_{50}$/ml increase) in IPNV-Thailand. All four salmonid sera inhibited IPNV-Thailand, while no inhibition was detected from non-salmonid sera. Salmonid sera tested showed high inhibition except BTS, while non-salmonid serum did not inhibit virus replication. In salmonid sera, IPNV Thailand isolates were highly inhibited by COS, CHS, and RTS with $10^{-4.7}$TCID$_{50}$/ml, $10^{-5.6}$ TCID$_{50}$/ml, and $10^{-7.8}$TCID$_{50}$/ml reduction each in virus titer. The isolate was only slightly inhibited by BTS with a $10^{-1.5}$TCID$_{50}$/ml virus reduction, however the inhibition level was statistically different than controls ($df=1$, $F=29.455$, $p=0.0056$). IPNV isolate Jasper was less inhibited by salmonid sera compared to the IPNV Thailand isolate. IPNV-Jasper was inhibited by RTS and COS with $10^{-6.5}$TCID$_{50}$/ml and $10^{-2.2}$TCID$_{50}$/ml reduction each; however, the isolate was not significantly inhibited by CHS (10^{0}TCID$_{50}$/ml reduction) or BTS ($10^{-0.6}$TCID$_{50}$/ml reduction) in which virus reduction level was not significant ($df=1$, $F=0.529$, $p=0.5072$).

Figure 4.10 Species range of serum inhibitory activity against 2 IPNV of different serotypes, Jasper (A_9) and Thailand (A_2), tested on the RTG-2 cell line. The activity of serum inhibition against the viruses was measured using seven sera from four salmonid and three non-salmonid fishes. Each serum is designated as follows: FLS (Starry Flounder Serum); SAS (Sablefish Serum); HES (Pacific Herring Serum); COS (Coho Salmon Serum); CHS (Chinook Salmon Serum); BTS (Brook Trout Serum); RTS (Rainbow Trout Serum). Inhibition of virus titer was measured seven days postexposure. Inhibition above $10^{1.0}$ is considered significant.

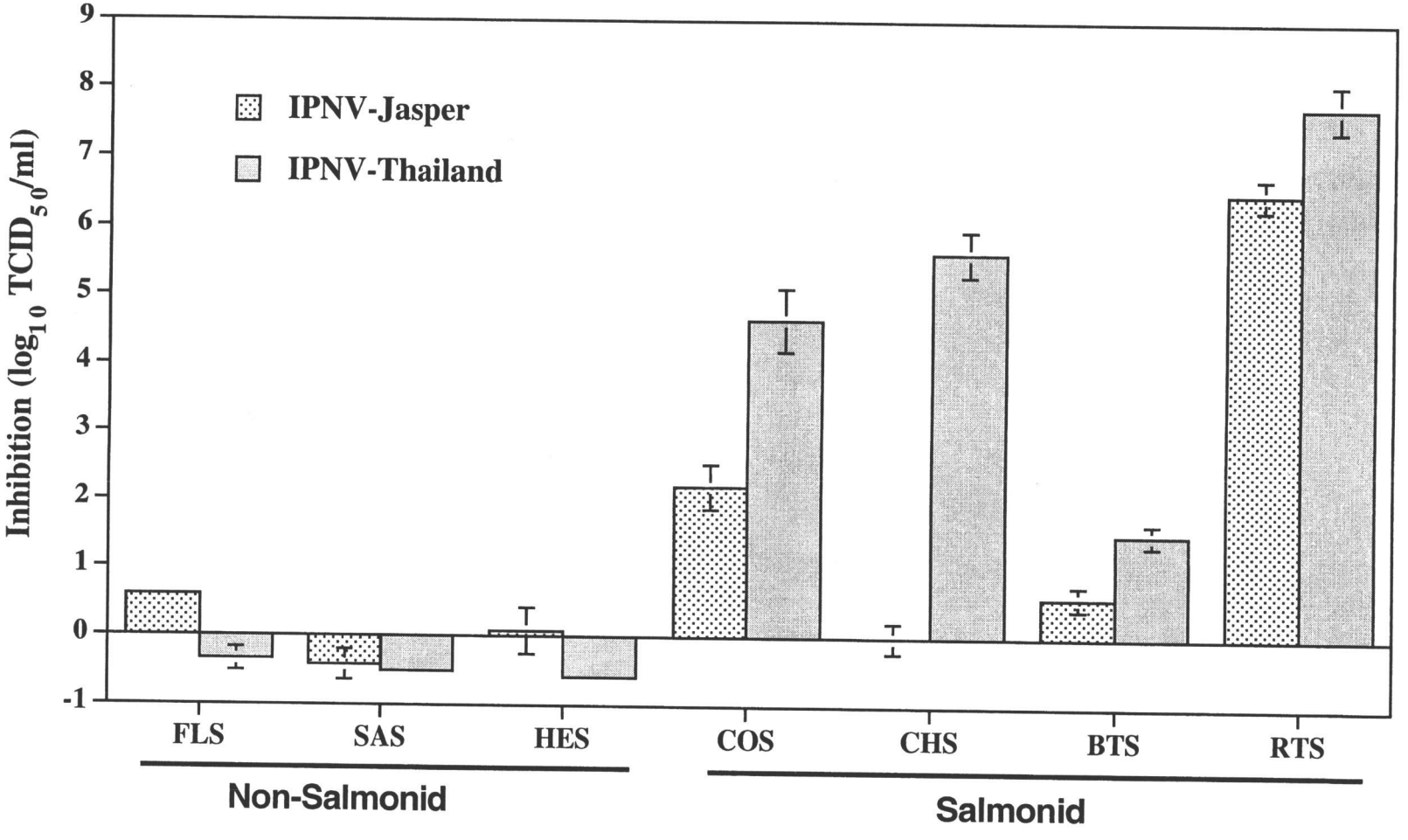
Inhibition (log_{10} $TCID_{50}$/ml)
9
8
7
6
5
4
3
2
1
0
-1
IPNV-Jasper
IPNV-Thailand
FLS
SAS
HES
COS
CHS
BTS
RTS
Non-Salmonid
Salmonid

Figure 4.10

DISCUSSION

Previous work has indicated that there is significant variation in RTS inhibition level depending up on the IPNV isolate used, but no information is available about the effects of virus passage history, cell line, cell density or other factors used in *in vitro* inhibition. These experiments were conducted to determine whether any of these factors could affect the ability of serum to inhibit IPNV replication. Ögut (1995) found that each IPNV isolate has different sensitivity to RTS. We also found significant differences in sensitivity to RTS depending on cell type or cell condition used for assaying inhibition. Hill & Dixon (1977) reported that sensitivity to RTS was developed sooner for IPNV serially passed in the EPC cyprinid line than in RTG-2 or BF cell lines. Kelly and Nielsen (1985) reported that susceptibility of IPNV-VR 299 from two separate laboratories to RTS was different; the virus from one laboratory was inhibited but the virus from the other laboratory was not. We also found sensitivity could be changed depending on the cells in which virus was replicated. Although Kelly & Nielsen (1985) reported inhibition in CHSE-214, we found the virus was sensitive to RTS when grown in RTG-2 cells, but not in CHSE during 7 days incubation (Figure 4.5). However, RTS-sensitive virus which had replicated in RTG-2 cells became less sensitive to RTS when passaged five times in CHSE cells (Figure 4.6). IPNV begins to produce large amount of progeny after at least 20h (Malsberger & Cerini 1963), and it is possible that CHSE-214-progeny virus is resistant to RTS inhibitor, or that interferon production in CHSE-214 is not high enough to inhibit IPNV replication. The reason for suggestion of interferon involvement is that we found virus inhibition differed on cell lines from salmonids and non-salmonids (Figure 4.8 & 4.9) and that RTS inhibition was high at high cell density (Figure 4.7).

Both RTG-2 and CHSE-214 cell lines have been known to become persistently infected by defective interfering particles (MacDonald & Kennedy 1979, Hedrick and Fryer 1981). However, they have difference in interferon production; RTG-2 cell line has been known to produce interferon (de Kinkelin & Dorson 1973; Okamoto et al 1983b), but not CHSE-214 (MacDonald & Kennedy 1979). MacDonald & Kennedy (1979) reported that persistent infection could be induced in CHSE-214 cells; however, no evidence of interferon-like activity was detected. The result of our experiment on the effect of RTS pretreatment of cells for 24h before infection (Figure 4.2) indicates that inhibition is not induced by cell pretreatment with RTS, such as interferon. Another result from our experiments confirmed that RTS inhibition was not related to masking of viral receptors on the cells (Figure 4.3). However, an interesting result was obtained from an experiment on the effect of cell density on RTS inhibition (Figure 4.7), in which higher cell densities, yielded higher inhibition by RTS. That result brought up two possibilities. The first was interferon involvement because interferon inhibition was stronger when RTG-2 cell density was high (Okamoto et al. 1983). Secondly, progeny virus replicated in high cell density is more sensitive to RTS. However, we found that there was no significant difference in RTS sensitivity between virus produced from low cell density of RTG-2 cells and from high cell density of RTG-2 cells (data not shown). It has been reported that IPNV induced interferon in both rainbow trout (Dorson et al. 1992) and cell lines from rainbow trout (de Sena and Rio 1975; Okamoto et al 1983b). Fathead minnow cells (FHM) (Gravell & Malsberger 1965; Oie & Loh 1971) and RTG-2 cells (de Kinkelin & Dorson 1973; Okamoto et al 1983b) have been well studied in this regard and it has been shown that they secrete interferon in reponse to viral infection. Interestingly these two

cell lines, FHM and RTG-2, have been most often used in the study of virus inhibition test *in vitro*. Okamoto et al (1983b) reported that inteferon production due to IPNV infection in RTG-2 cells was dependent on cell densities; interferon production was high on tightly confluent cell monolayers while it was low on loosely confluent cell monolayers. It was also found that viral infectivity titers were noticeably decreased on 3 day old cell monolayer showing a tightly confluent monolayer. They interpreted low viral infectivity titer to be a consequence of high interferon production. We also found that inhibition by RTS was high on a tightly confluent cell monolayer while there was no inhibition on a loosely confluent cell monolayer (Figure 4.7).

Neither RTG-2 nor CHSE-214 cells showed CPE until three days after exposure to IPNV (data not shown). However, three days later, CPE in CHSE-214 rapidly appeared, and ultimately these cells produced the same virus titer in MEM-10 and 1% RTS as in MEM-10. By contrast, virus grown in RTG-2 cells in the presence of 1% RTS showed no evidence of CPE. In general, CPE development in RTG-2 was dependent on serum and cell density. At a low cell density (loosely confluent), CPE developed in 5 to 7 days, while at a high cell density (tightly confluent), CPE was not apparent developed 7 days or even 14 days postinfection. However, CPE development did not always followed this pattern but was dependent on serum source and freshness of RTS. CHS did not significantly inhibit IPNV replication in CHSE-214 cells or in RTG-2 cells (Figure 4.5); however, we found that higher inhibition was obtained in CHSE-214 cells than in RTG-2 cells. This indicates that the lack of inhibition by RTS in CHSE-214 is partially related to serum-host specificity as well as to the cell line.

We found that sensitivity of IPNV to RTS changes. Virus has same RTS sensitivity among two cell lines, RTG-2 and CHSE-214, at the first exposure to RTS before viral attachment to cell. However through viral replication, virus produced in CHSE-214 become RTS resistant in the process of viral passages. Therefore, we questioned whether RTG-2 and CHSE-214 have different characteristics of cell membrane and thus, IPNV conduct viral modification such as with envelope-type virus. The possibility of glycosylation in IPNV has been suggested (Estay et al. 1990, Hávarstein et al. 1990, Hjalmarsson et al. 1999). Hjalmarsson et al. (1999) reported that the capsid protein VP2 of IPNV was glycosylated. In another study, we observed that IPNV isolate Jasper, which is sensitive to RTS inhibition ($\geq 10^{-6}$/ml $TCID_{50}$), replicated in the presence of RTS produced heterogeneous progeny with respect to RTS sensitivity (Park unpublished results). Approximately 10% of clones tested were RTS resistant. We found a high genetic difference in VP2 region between RTS sensitive clone and RTS resistant clone. This RTS–resistant clone was passaged once more in RTG-2 and kept RTS resistant. Thus, quantitative composition between RTS sensitive and RTS resistant clones will be important factor in RTS inhibition experiment *in vitro* and *in vivo*.

Even though it has been reported that inhibition by RTS occurred also in non-salmonid cell lines, FHM (Kelly & Nielsen 1985), BF-2 and EPC (Hill & Dixon 1977), We found that RTS was toxic to FHM and EPC cell lines even at 1:1000 RTS dilution and RTS inhibition was insignificant in BF-2 cells and PHE-184. However, we found significant RTS inhibition in CCO and BB cell lines (Figure 4.9). The difference between the result of Hill & Dixon (1977) and our result about the RTS inhibition in BF-2 might be caused by method difference used in experiments. They used the plaque assay method

allowing approximately 2-4 days of incubation time, while we allowed 7 days of viral replication time for RTS inhibition. As in inhibition comparison between RTG-2 and CHSE-214 (Figure 4.5), we suspect that initially IPNV in BF-2 cells might be inhibited by RTS, however the virus was not further inhibited during 7 day incubation.

All information about trout serum inhibition has been obtained from rainbow trout serum (Vestergard-Jörgensen PE 1973, Dorson & Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978, Kelly & Nielsen 1985, Ögut 1995). In our studies, it was found that serum inhibition was associated only with salmonid sera but not with non-salmonid sera (Figure 4.10). Although three of four salmonid sera tested showed strong inhibition, brook trout serum (BTS) showed slight inhibition against IPNV-Thailand and no inhibition against IPNV-Jasper. This result is meaningful in IPNV disease because brook trout is known to be the most susceptible species for IPNV (Silim et al. 1982). Thus, resistance of IPNV to RTS inhibitor could be a virulence factor. For the experiment of species range of serum inhibitory activity against IPNV, we used salmonid cell line, RTG-2. However, this indicates that no inhibition of RTS inhibitor in non-salmonid sera is not caused by serum-host cells specificity. The reason is herring serum did not inhibit IPNV-Jasper in host cells, PHE-184 (data not shown). Even among the salmonid cell lines, some cell lines showed RTS inhibition; however, some cell lines did not (Figure 4.8). These above results could come about by two possible mechanisms: one is that serum inhibitor ("6S") against IPNV is present in only salmonid sera and each fish species has different amount of serum inhibitor, and the other is that serum induces inhibition produced by cell line, which is cell line dependent. Even though we can not

perfectly exclude serum-host cells specificity, we believe that salmonid serum has higher serum inhibitor against IPNV than that of non-salmonid serum.

Kelly & Nielsen (1985) determined the effect of trout serum on $_{32}$P-labeled IPNV-Sp adsorption. They observed that approximately 97% of IPNV-Sp was not adsorbed to the FHM cells in the presence of RTS, whereas about 55% of control virus was unadsorbed to cells. In our experiment on the effect of 2h pretreatment with RTS we found that more IPNV was inactivated by only 2hr treatment with RTS ($10^{-4.5}$ $TCID_{50}$/ml reduction from $10^{8}TCID_{50}$/ml) and $10^{-7.0}TCID_{50}$/ml reduction when RTS was present during viral replication for 7 day. Even though our experiment and the experiment of Kelly & Nielsen (1985) were conducted by different methods, there was a common observation that IPNV is directly inhibited by RTS and some portion of the virus could adsorbed to cells even in the presence of RTS. We found that RTS should be present during viral replication to inhibit the replication of penetrated virus. For the further RTS inhibition in 7 days incubation, it will be more dependent on cell line used rather than serum-host cells specificity. Thus, phenotypic characteristics of progeny virus and the amount of interferon production from cells could be important factor for latter RTS inhibition in 7 days incubation.

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CHAPTER 5

THE EFFECT OF *IN VITRO* PASSAGE OF INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV) ON VIRULENCE AND SENSITIVITY OF THE VIRUS TO RAINBOW TROUT SERUM (RTS)

Kyoung C. Park and Paul W. Reno

ABSTRACT

Three IPNV isolates (A_1 serotype, Buhl subtype) were passaged five times in RTG-2 cells with either MEM-10 or MEM-10 with 1% rainbow trout serum (MEM-RTS), to determine the effect of passage of IPNV on virulence and sensitivity of the virus to RTS. Mortality level in brook trout fry was highly variable during viral passages; however, in general, most of IPNV isolates under all conditions were virulent. Mortality level by IPNV isolates ranged between 30-89%. Sustenance of virulence during viral passage five times *in vitro* was dependent on IPNV isolate and culture conditions. Even one passage at different condition, *in vitro*, highly effect on virulence. RTS did not always help to keep viral virulence during five times *in vitro* passages. The peak epizootic by three isolates after multiple passages was virulence dependent: high virulence isolate showed peak mortality from 4 to 11 days post-exposure, while low virulence isolate showed delayed peak mortality from 8 to 14 days post-exposure. IPNV isolates passaged in MEM-10 or MEM-RTS was over all resistant to inactivation by RTS *in vitro* except for IPNV isolate crayfish passaged in MEM-10 which became highly sensitive to RTS showing up to $10^{-8.0}$ $TCID_{50}$/ml reduction in virus titer. However, we found highly increased RTS sensitivity of the virus was not correlated with decreased virulence. We found over all that passage in the presence of RTS does not affect virulence and that no relationship was obtained between virulence and RTS sensitivity of the virus. All three isolates showed identical antigenicity patterns with a panel of 11 monoclonal antibodies, irrespective of viral passage conditions.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV), belonging to the family birnaviridae, was the first virus isolated from teleosts (Wolf *et al.*, 1960) and can cause mortality as high as 90-100%, in 1-4 month-old rainbow trout *Oncorhynchus mykiss* (Frantsi & Savan 1971, McAllister 1983). Serum from unexposed rainbow trout (RTS) inhibited IPNV replication *in vitro* (Vestergard-Jörgensen 1973, Dorson & Kinkelin 1974). The serum inhibitor was estimated to have a sedimentation coefficient of approximately 6S by ultracentrifugation and thus is different from fish antibody, IgM, which has a sedimentation coefficient of 14-16S (Dorson & de Kinkelin 1974). Ögut (1995) tested the ability of RTS to inactivate 109 IPNV isolates, from all serogroup A serotypes (Hill & Way 1995): A_1=WB; A_2=Sp; A_3=Ab; A_4=He; A_6=Canada1; A_7=Canada2; A_8=Canada3; A_9=Jasper, and 65 IPNV isolates were inactivated (greater than 10^{-1} $TCID_{50}$/ml reduction) by RTS. However, even within one serotype (A1=WB), not all virus isolates tested were inhibited by RTS. Cell culture-adapted virus strains were more susceptible to RTS than were wild type virus (Dorson & Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978). Kelly & Nielson (1985) determined that virus adsorption to cells is reduced in the presence of RTS. Approximately 97% of virus pre-incubated with serum remained unadsorbed, whereas about 55% of control virus did so.

Since the first report relating the ability of a 6S serum component to inhibit replication of IPNV, it has been suggested by some researchers that the ability of IPNV strains to replicate in the presence of normal trout serum is an important aspect of virus pathogenesis and can be considered as a virulence factor (Hill & Dixon 1977, Hill 1982). It has been reported that 6S sensitivity was correlated with virulence of IPNV and that

avirulent strains could become virulent by passaging virus in the presence of RTS (Hill and Dixon 1977, Hill 1982, Ögut 1995) although some researchers found opposite results (Dorson et al 1975, Dorson et al 1978, MacAllister and Owens 1986). The published information thus indicates that 6S resistance does not always correlate with virulence and that RTS does not always sustain viral virulence during viral passage *in vitro*. The present study was carried out to clarify the potential effect of *in vitro* passage of IPNV on rainbow trout serum sensitivity and virulence. Although earlier reports compared the change of RTS sensitiviry or virulence during viral passages with RTS or MEM-10 (Dorson et al. 1975; Hill & Dixon 1977; McAllister & Owens 1986; Ögut 1995), no information is available comparing the relationship between RTS sensitivity and virulence at each passage during multiple passages. In addition, serologically different IPNV strains (WB vs Sp) were used in various studies (Dorson et al. 1975; Hill & Dixon 1977; McAllister & Owens 1986). Thus it was difficult to combine the information about the effect of viral passage on the relationship between 6S sensitivity and virulence. Another question we attempted to answer is if the change in RTS susceptibility during viral passages *in vitro* is related to epitope alteration. Thus, in this paper, we report the effect of passage of virus, three closely related Buhl subtype strains which were RTS sensitive or RTS resistant.

MATERIAL AND METHODS

Virus passage *in vitro*

All three IPNV isolates were serotype A_1, subtype Buhl. All isolates were from trout culture facilities in Idaho. Isolates 90-11 and 183-82 were originally isolated from healthy and diseased rainbow trout respectively; isolate "crayfish" was from *Astarus astarus* upstream from an aquaculture facility undergoing an IPNV epizootic. These stocks of viruses were isolated from experimentally infected brook trout (*Salvelinus fontinalis*) then passaged once in cell culture (CHSE-214 cells; Lannan et al 1984) and had been stored at -80°C until use. In these experiments the isolates were passaged once in brook trout weighing 0.5g. Upon reisolation from dead fish (passage 0), the viruses were passaged five times (passage 1 to passage 5) in RTG-2 cells (Wolf and Quimby 1962) in 75 cm^2 tissue culture flasks with minimum essential medium containing either 10% fetal bovine serum (MEM-10) or containing 10% fetal bovine serum and 1% rainbow trout serum (MEM-RTS). The three isolates passaged in RTG-2 cells were held at -80°C until they were used for RTS sensitivity tests and virulence tests.

Rainbow trout serum

Rainbow trout were obtained from Alsea Hatchery, Alsea, OR. Fish health inspection indicates that there has been no IPNV detected at this facility for more than 25 years. Blood samples were collected by caudal vein puncture and pooled from 20 adult fishes weighing approximately 600 g each. These samples were allowed to clot at 5°C

overnight and centrifuged at 1000xg for 20min. The serum was collected and portions were dispensed into 1 ml aliquots and stored in liquid nitrogen until use.

In vitro virus sensitivity to RTS

The RTG-2 cells (Wolf and Quimby 1962) were propagated in 24 well microtiter plates as described by Caswell-Reno et al (1989). Three replicate wells were prepared for each condition . In this experiment, three types of media were used: MEM without serum=MEM-0; MEM+10% fetal bovine serum=MEM-10; MEM+10% fetal bovine serum+1%RTS=MEM-RTS. Virus was diluted with MEM-10 or MEM-RTS to give the final virus concentration of $10^4 TCID_{50}$/ml. Three hundred μL of diluted virus was added onto a confluent monolayer in each well and incubated for two hours. After the incubation time, the inoculated monolayers were washed three times with MEM-0 and then 1ml of either MEM-10 or MEM-RTS was added. Uninoculated controls were treated with either medium MEM-10 or MEM-RTS as appropriate. Cells were incubated at 18°C for seven days in an incubator to which 5% CO_2 was supplied. The cytopathogenic effect (CPE) was monitored daily and scaled on a 0 (no CPE) to 4 (complete CPE) ordinal scale. On the seventh day, virus from each well were harvested and kept in liquid nitrogen until they were titrated.

Virulence

Brook trout were obtained from Wizard Falls Hatchery, Camp Sherman, OR. IPNV has not been detected at this hatchery since 1976. The fish were held at the laboratory for Fish Disease Research at Hatfield Marine Science Center, Newport, Oregon in dechlorinated city water. Fish weighing 0.76g were placed into 7L tanks containing 5L of water at 12°C at a flow rate of 1L/min. Two replicates were used for each treatment (or passage) and five tanks were used for controls. The final concentration of virus added to each tank was $10^4 TCID_{50}$/ml. Fish were exposed to virus in static water for five hours with aeration. For the control tanks, same amount of MEM-10 (5ml) was added to emulate the test concentration of medium (McAllister & Owens 1986). After five hours, clean water was supplied with an approximate flow rate of 1L/min. Fish were monitored daily for signs of IPN disease; dead fish were collected daily and cumulative mortality was recorded over a 30 day period. The collected whole fish were held in liquid nitrogen until assayed to determine viral presence.

Statistical analysis

Mortality patterns using survival analysis at each passage were compared. The Kaplan-Meier (product-limit) method was used to evaluate differences in survival patterns among groups of each passages. Survival Tools for StatView (Abacus Concepts, Inc., Berkeley, CA, 1994) was used to perform all these analyse.

Enzyme immunodot assay

To confirm epitope stability, an enzyme immunodot assay was performed on three isolates passed *in vitro* following the procedure of Caswell-Reno et al. (1989). Briefly nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA) was soaked in Tris-buffered saline (TBS), pH 7.5 for 30 min and then placed into a 96 well immunodot apparatus (Bio-Rad). One hundred µl of each passage virus (10^7 $TCID_{50}$/ml) was added into each horizontal row of 12 wells on the apparatus. After a 2hr adsorption time, 100 µl of 3% bovine serum albumin (BSA) in TBS were added and allowed for one hour to block unbound sites on the membrane. After washing three times with 200 µl of TBS, 100 µl of each monoclonal antibody (Mabs) were added to appropriate wells and incubated for one hour. After washing three times with TBS, 100 µl of a 1:1000 dilution of goat anti-mouse IgG (conjugated with horseradish peroxidase) was added to each well and incubated for one hour. After washing three times, the membrane was transferred to a tray and washed one more time with TBS. The membrane was incubated with substrate (1.7 ml of 0.3% 4-chloronaphthol in 100% methanol, 100µl of 3% hydrogen peroxide, and 8.2 ml of TBS). Reaction was stopped by washing three times with TBS when the background began to turn purple. Positive reactions were indicated by a purple color. Supernatant collected from uninfected CHSE-214 cell culture was used as a negative-control antigen.

RESULTS

Virulence

To determine the effect of multiple *in vitro* passage of IPNV on virulence to fish, we tested virulence of all isolates passaged under different conditions. In general, all isolates of viruses under all conditions were virulent in 0.76g brook trout. Cumulative mortality reached 89% even though unusually low mortality (3%) was obtained from isolate 90-11 passage 5 in MEM-10; virus at low titer was isolated from these fish. Average control mortality was 0.2%. Behavioral signs of IPNV disease were observed in infected fish and no virus was detected from control fish. Cumulative mortality from isolates 90-11, crayfish, and 183-82, was variable dependant on culture condition or viral passage; 3-59% for IPNV isolate 90-11 (Figure 5.1), 38-89% for IPNV isolate crayfish (Figure 5.2), 38-72% for IPNV isolate 183-82 (Figure 5.3).

The cumulative mortality of all passages of IPNV isolate 90-11 is shown (Figure 5.1) and the comparison of the Kaplan-Meier (nonparametric) survival analysis of each passage or each passage type of the isolate is shown in Table 5.1. The first mortality occurred 3 days post-exposure from the virus passaged once in MEM-RTS and mortalities were observed by 7 days post exposure for all *in vitro* passages. Cumulative mortality level was highly variable during viral passages; 3-59% for passage in MEM-10 and 34-57% for passage in MEM-RTS. IPNV isolate 90-11 showed no significant difference in mortality level during *in vitro* passages in MEM-10 or MEM-RTS until passage 4 (Table 5.1). At passage 5, however, the mortality level was significantly decreased to 3% in MEM-10 and 34% in MEM-RTS. A comparison of survival between

passage 1 and passage 5 was highly significant (Table 5.1) and survival rates between fish exposed to isolate 90-11 passage 5 in MEM-10 and MEM-RTS was also highly significant (χ^2=32.126, df=1, p<0.0001). The dynamics of IPNV disease also changed with passage. The median day to death was 6 for passage 1 in MEM-10 and 13 for passage 5; likewise 5 to 11 in MEM-RTS.

The mortality data for brook trout exposed to all passages of IPNV isolate crayfish are shown in Figure 5.2 and the comparison of survival analysis of each passage or each passage type of the isolate is shown in Table 5.1. In most cases, mortality from IPNV-crayfish started in the range of 3-7 dpe. Mortality by IPNV crayfish showed high variation and was higher in MEM-10 than MEM-RTS during 5 *in vitro* passages; 65-89% in MEM-10 passages, 38-80% in MEM-RTS passages. It was found from IPNV-crayfish that a single *in vitro* passage at different condition highly effect on virulence. The isolate passaged with MEM-10 showed higher virulence than the isolate passaged with MEM-RTS from all passages. At passage 1, the isolate passaged in MEM-10 or MEM-RTS showed similar mortality level (χ^2=2.295, df=1, p=0.1298). However, passage2 in MEM-RTS showed highly decreased virulence and this decreased virulence continued until passage 5. The pattern of survival analysis between MEM-10 and MEM-RTS from passage 2 was statistically very different (χ^2=68.990, df=1, p<0.0001). This statistical difference was continuous until passage 5. The virulence of virus passaged in MEM-10 was always higher than the virulence of virus passaged in MEM-RTS. Passages 2, 3, 4 of MEM-10 showed similar virulence to the virulence of passage 1 in MEM-10: however, survival analysis showed that passages 2, 3, and 4 were statistically different to passage 1 (Table 5.1).

The epizootic pattern of IPNV- crayfish passaged in MEM-10 was generally virulence dependent. The virus showing high mortality, passage 1, 2, 3, and 4 showed similar pattern: peak epizootic was occurred from approximately 4 to 11 days post-exposure. However, passage 5 having lowest virulence showed delayed peak epizootic from 8 to 14 days post-exposure, peak daily mortality was occurred on different day during the epizootic period among each passages.

The isolate crayfish passaged in MEM-RTS showed three types of epizootic pattern. The most virulent virus, passage 1 showed early epizootic from 4 to 9 days post-exposure; while low virulent passage 2, 4,and 5 showed delayed epizootic from 7 to 14 days post-exposure. Another low virulence virus passage 3 showed distinct epizootic pattern among other passages. This passage also showed delayed epizootic: first one occurred from 11 to17 days post-exposure and second one occurred from 20 to 23 days post-exposure.

The mortality of all passages of IPNV isolate 183-82 is shown in Figure 5.3 and the comparison of survival analysis of each passage or each passage type of the isolate is shown in Table 5.1. Mortality started on 3 days post-exposure. The cumulative mortality ranged between 38-72% during viral passages; 38-65% in MEM-10 and 39-72% in MEM-RTS.

Passage 1 in MEM-RTS showed similar mortality pattern compared to passage 1 in MEM-10 (χ^2=0.270, df=1, p=0.6036). This similar mortality pattern between MEM-10 and MEM-RTS was continue until passage 5, except in passage 3 (χ^2=9.619, df=1, p=0.0019). Passage 3 in MEM-10 showed a decreased virulence compared to passage 3 in MEM-RTS. Among MEM-10 passages, passage 2, 4, and 5 showed similar mortality

pattern to passage 1 in MEM-10 (Table 5.1): 49-65% mortality and peak epizootic from 4 to 10 days post-exposure. However, passage 3 had the lowest mortality compared to passage 1 (χ^2=12.616, df=1, p=0.0004) and there was delayed epizootic which occurred from 9 to 14 days post-exposure. Survival analysis indicated that passage 1, 3, and 4 of MEM-RTS passage were similar but that passage 2 and passage 5 were different from pasage1 (Table 5.1). Passage 5 also showed delayed epizootic.

In vitro virus sensitivity to RTS and its relationship to virulence

In order to determine if the sensitivity of IPNV isolates to RTS is changed by multiple viral passages and if this change affects viral virulence, we determined in vitro RTS sensitivity of each passage and compared it with virulence.

IPNV isolate 90-11 was resistant to inactivation by RTS after primary isolation (passage 0). IPNV isolate 90-11 passaged in MEM-10 or MEM-RTS was resistant to inactivation in MEM-RTS, except for passage 5 in MEM-RTS (Figure 5.4). After 5 passages in RTG-2 cells with MEM-RTS, the virus showed a $10^{-3.4}$ $TCID_{50}$/ml reduction in the presence of RTS compared to the virus titer in MEM-10. This increased RTS sensitivity was correlated with decreased mortality (Figure 5.1). However, passage 5 in MEM-10, showing RTS resistance, showed a greater decrease in mortality when compared to passage 5 in MEM-RTS. Although the passage 4 in MEM-10 also showed $10^{-1.2}$ $TCID_{50}$/ml reduction, this reduction was minimal; all other passages showed RTS resistance.

Figure 5.1 Cumulative mortality in brook trout fry (0.76g) exposed to each passage of IPNVisolate 90-11 after five times passage in RTG-2 cells with MEM-10 (A) or MEM-RTS (B). Brook trout fry were immersed with the final concentration of $10^4 TCID_{50}$/ml IPNV 90-11 for five hours.

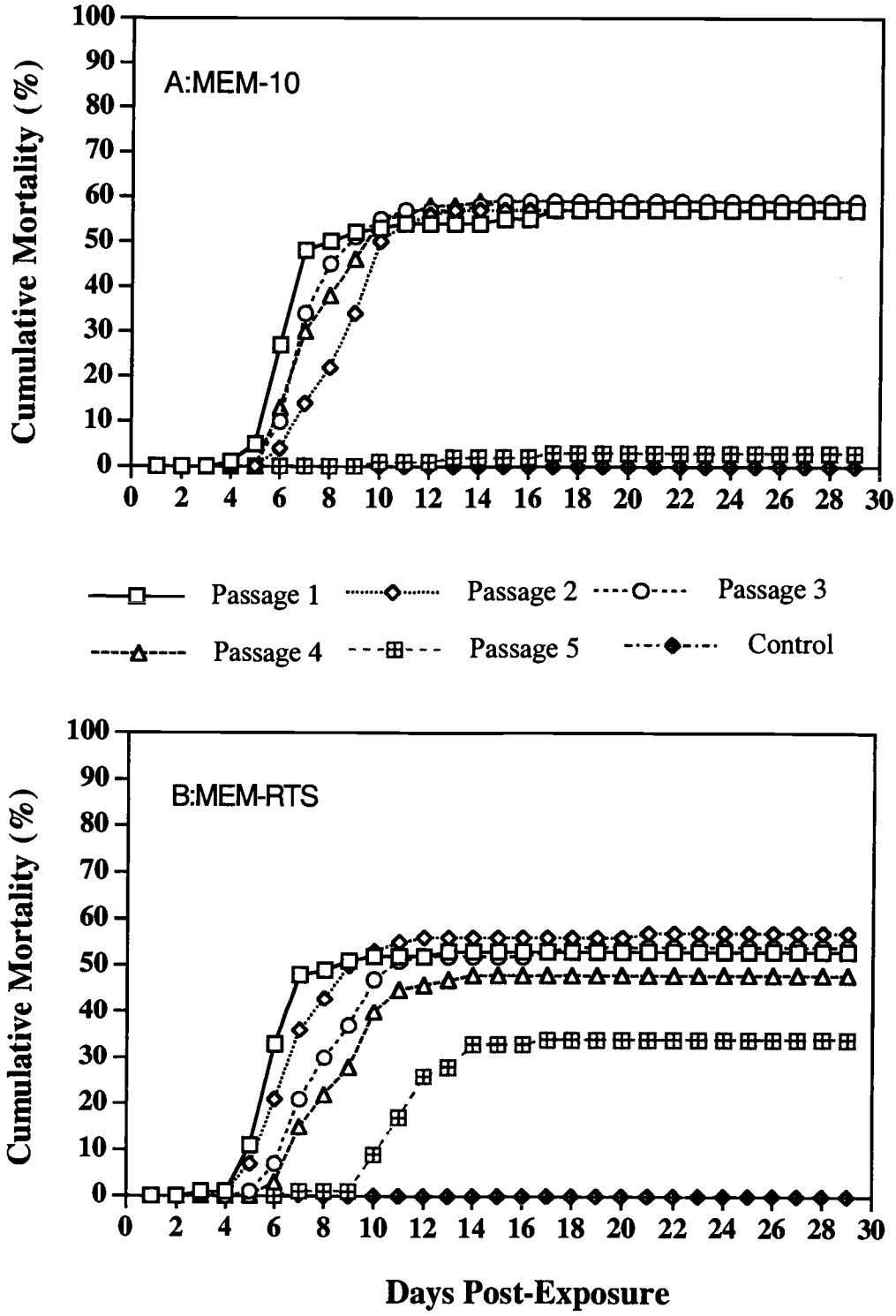
A:MEM-10
Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5
Control
B:MEM-RTS
Cumulative Mortality (%)
Days Post-Exposure

Figure 5.1

Figure 5.2 Cumulative mortality in brook trout fry (0.76g) exposed to each passage of IPNV isolate crayfish after five times passage in RTG-2 cells with MEM-10 (A) or MEM-RTS (B). Brook trout fry were immersed with the final concentration of $10^4 TCID_{50}$/ml IPNV crayfish for five hours.

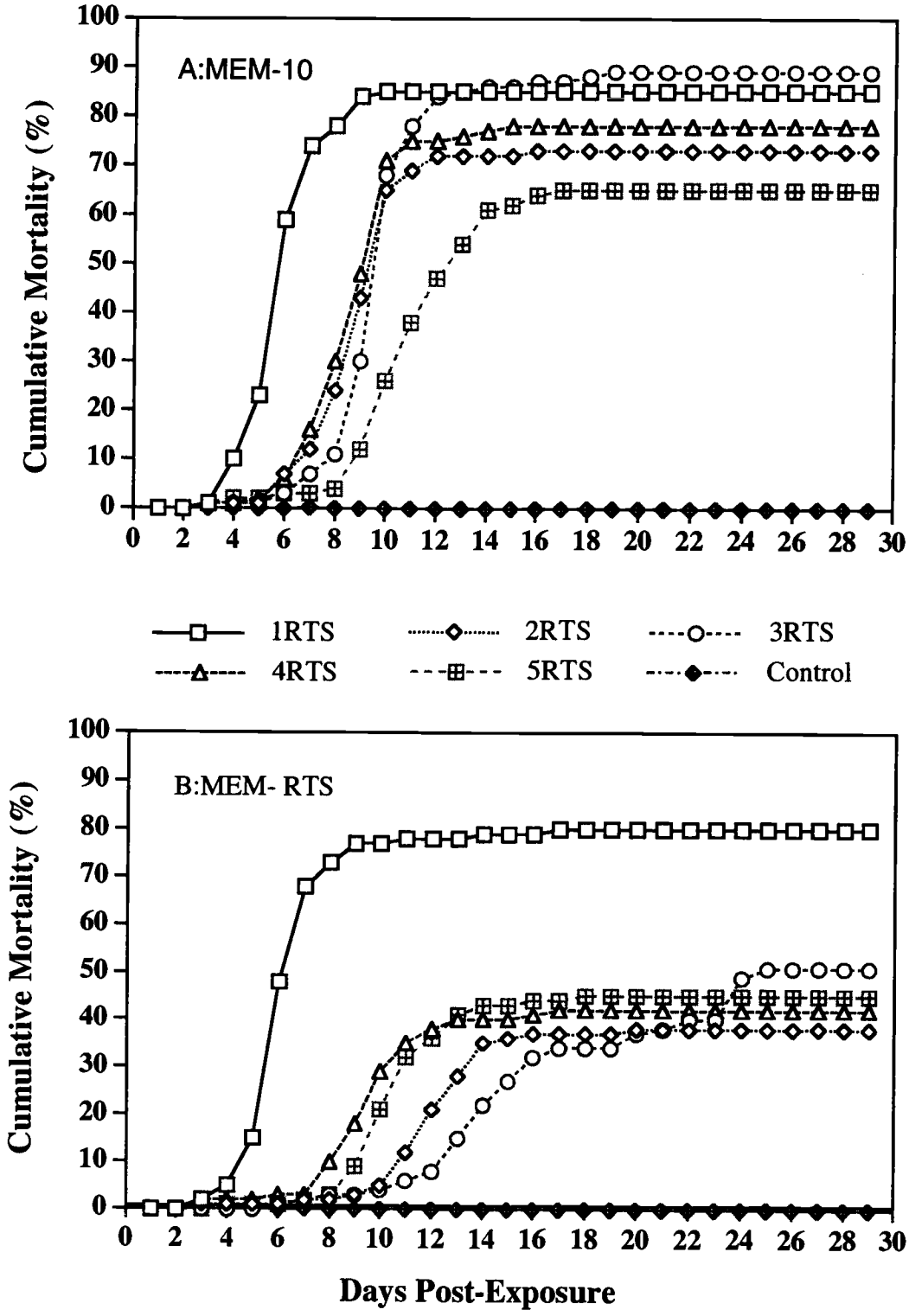
A:MEM-10
Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
1RTS
2RTS
3RTS
4RTS
5RTS
Control
B:MEM- RTS
Cumulative Mortality (%)
Days Post-Exposure

Figure 5.2

Figure 5.3 Cumulative mortality in brook trout fry (0.76g) exposed to each passage of IPNVisolate 183-82 after five times passage in RTG-2 cells with MEM-10 (A) or MEM-RTS (B). Brook trout fry were immersed with the final concentration of $10^4 TCID_{50}$/ml IPNV 183-82 for five hours.

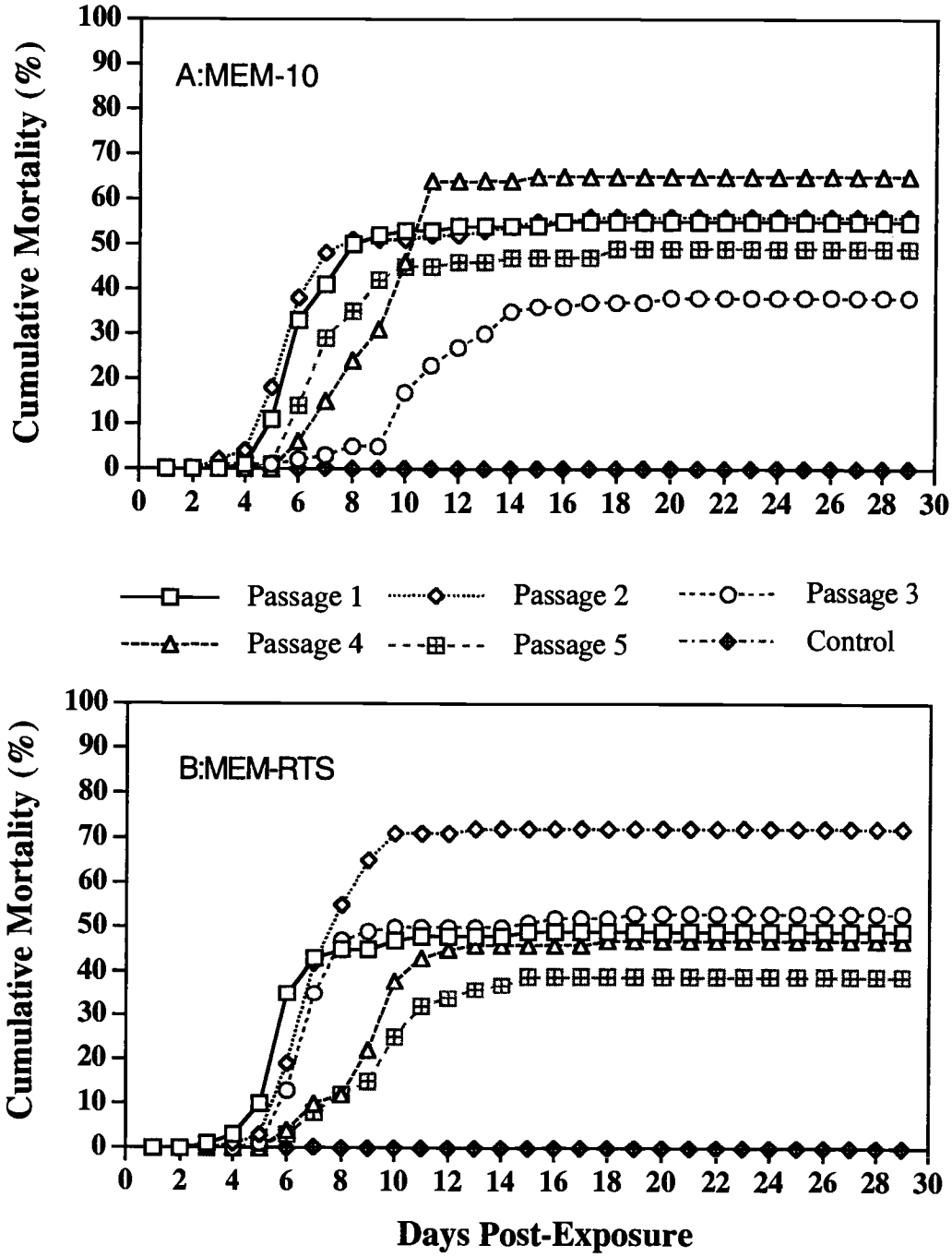
A:MEM-10
B:MEM-RTS
Cumulative Mortality (%)
Days Post-Exposure
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5
Control

Figure 5.3

Table 5.1 Comparison of survival analysis of each passage or each passage type from three IPNV isolates, 90-11, crayfish, and 183-82 which passaged five times in RTG-2 cell line with MEM-10 or MEM-RTS. Each passage type in comparison of survival analysis designate as follows: MEM[a]=MEM-10; RTS[b]=MEM-RTS. $p \geq 0.05$ was considered statistically no difference in viral virulence. In all survival analysis, degrees of freedom (df) was 1.

Passage number or passage type	Comparison of survival analysis	IPNV isolate 90-11		IPNV isolate crayfish		IPNV isolate 183-82	
		χ^2	p-value	χ^2	p-value	χ^2	p-value
Passage 1	MEM[a] vs. RTS[b]	0.001	0.9713	2.295	0.1298	0.270	0.6036
Passage 2	MEM vs. RTS	1.167	0.2801	68.990	< 0.0001	1.097	0.2949
Passage 3	MEM vs. RTS	1.227	0.2679	83.250	< 0.0001	9.617	0.0019
Passage 4	MEM vs. RTS	3.997	0.0456	33.324	< 0.0001	6.114	0.0134
Passage 5	MEM vs. RTS	32.126	< 0.0001	6.915	0.0085	3.886	0.0487
MEM-10	Passage1 vs. Passage2	1.939	0.1638	33.451	<0.0001	0.284	0.5942
MEM-10	Passage1 vs. Passage3	0.379	0.5383	26.947	<0.0001	12.616	0.0004
MEM-10	Passage1 vs. Passage4	0.325	0.5689	29.278	<0.0001	0.056	0.8123
MEM-10	Passage1 vs. Passage5	74.609	< 0.0001	53.964	< 0.0001	2.507	0.1134
MEM-RTS	Passage1 vs. Passage2	0.033	0.8567	68.513	< 0.0001	4.379	0.0364
MEM-RTS	Passage1 vs. Passage3	1.392	0.2381	51.514	< 0.0001	0.159	0.6901
MEM-RTS	Passage1 vs. Passage4	3.934	0.0473	55.732	< 0.0001	2.531	0.1116
MEM-RTS	Passage1 vs. Passage5	17.395	< 0.0001	56.306	< 0.0001	6.697	0.0097

The IPNV isolate crayfish showed a very distinct pattern of RTS sensitivity (Figure 5.5). Virus passaged in MEM-10 became highly sensitive to RTS, even after primary isolation, losing $10^{-8.0}$TCID$_{50}$/ml in passage 4. However, no inhibition occurred when the IPNVisolate crayfish was passaged in MEM-RTS. Although crayfish mortality in MEM-RTS passages (Figure 5.2) was significantly decreased after one passage in MEM-RTS, that was not correlated with RTS sensitivity results (Figure 5.5). And also there was no correlation between RTS sensitivity and mortality after passage in MEM-10. Virus passage 2 through passage 5 in MEM-10 had significantly increased RTS sensitivity ($10^{-4.7}$-$10^{-8.0}$ TCID$_{50}$/ml reduction) than passage 1; however, this did not induce decreased mortality.

IPNV isolate 183-82 passaged in MEM-10 or MEM-RTS was not inhibited by RTS *in vitro* (Figure 5.6). However mortality result showed wide range of virulence, from 38%-72%, during multiple passages (Figure 5.3).

Enzyme immunodot assay

In order to determine if virus antigenicity was altered during passages *in vitro*, a panel of monoclonal antibodies was used. All three isolates passaged in MEM-10 or MEM-RTS showed identical monoclonal antibody reactions during each of five passages (Table 5.2). All showed typical Buhl subtype patterns showing positive reaction to AS-1, W3, W5, E1, E5, and E6. There were no differences in monoclonal reactions between RTS sensitive and RTS resistant virus. The same monoclonal reaction patterns were shown between sensitive crayfish isolate (passage 2, 3, 4, 5 in MEM-10) and resistant

crayfish isolate to RTS (passage 1, 2, 3, 4, and 5 in MEM-RTS). IPNV 90-11 isolate also showed identical monoclonal reactions between the isolate sensitive to RTS (passage5 in MEM-RTS) and all other passages showing RTS resistant.

Figure 5.4 The effect of *in vitro* passage on sensitivity of IPNV 90-11 to RTS inhibitiory activity *in vitro*. The virus was passaged five times with MEM-10 or MEM-RTS. Each of passaged virus with final concentration of $10^4 TCID_{50}$/ml in MEM-10 or MEM-RTS was infected to RTG-2 cells. Cells were incubated with either medium MEM-10 or MEM-RTS as appropriate during seven days. On the seventh day, virus titer from each medium was compared to determine RTS inhibition. The X-axis represents the passage number of IPNV 90-11 under each condition. The Y-axis represents the RTS sensitivity of each virus.

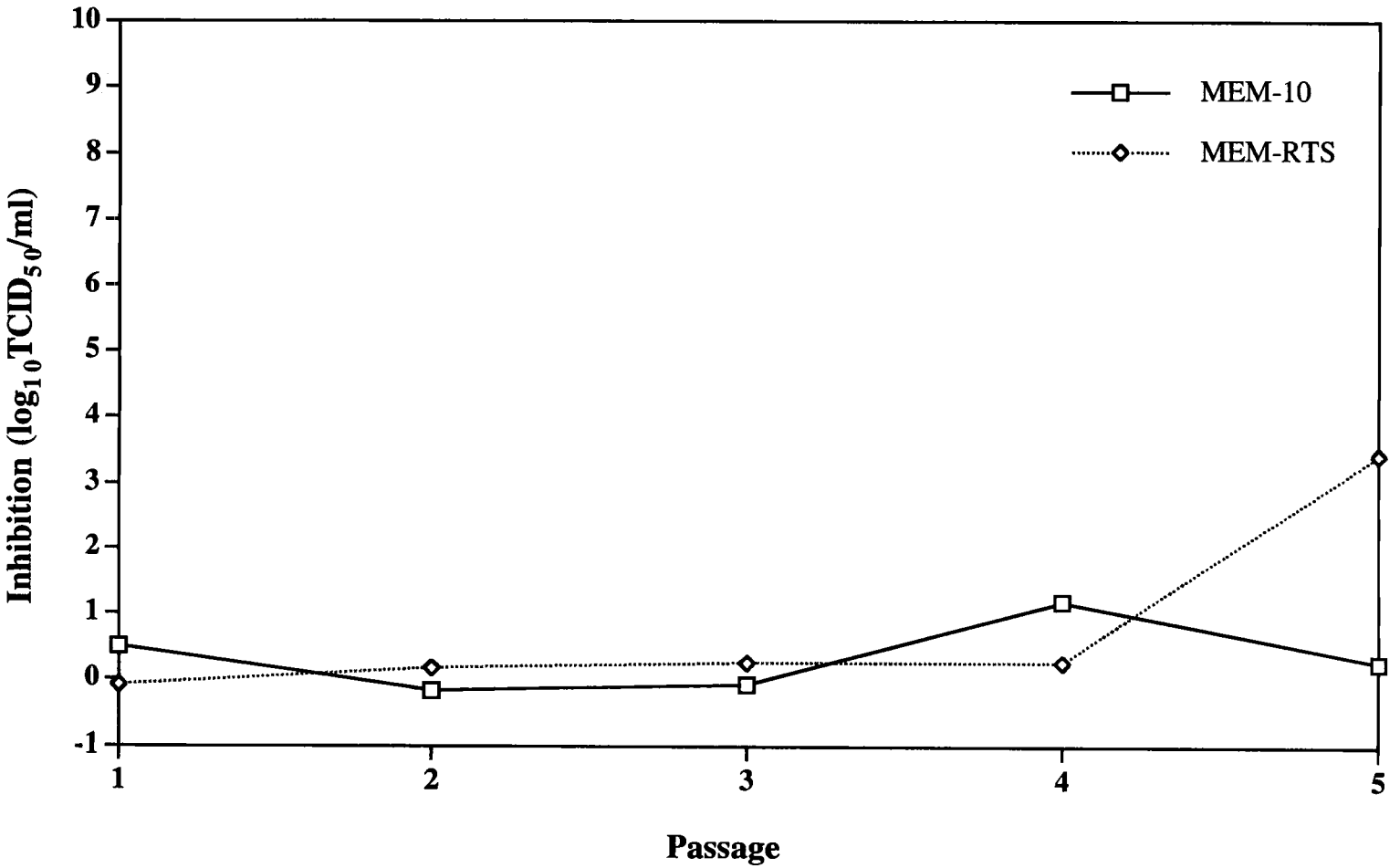
10
9
8
7
6
5
4
3
2
1
0
-1
1
2
3
4
5
Inhibition ($log_{10}TCID_{50}$/ml)
Passage
MEM-10
MEM-RTS

Figure 5.4

Figure 5.5 The effect of *in vitro* passage on sensitivity of IPNV crayfish to RTS inhibitory activity *in vitro*. The virus was passaged five times with MEM-10 or MEM-RTS. Each of passaged virus with final concentration of $10^4 TCID_{50}$/ml in MEM-10 or MEM-RTS was infected to RTG-2 cells. Cells were incubated with either medium MEM-10 or MEM-RTS as appropriate during seven days. On the seventh day, virus titer from each medium was compared to determine RTS inhibition. The X-axis represents the passage number of IPNV crayfish under each condition. The Y-axis represents the RTS sensitivity of each virus.

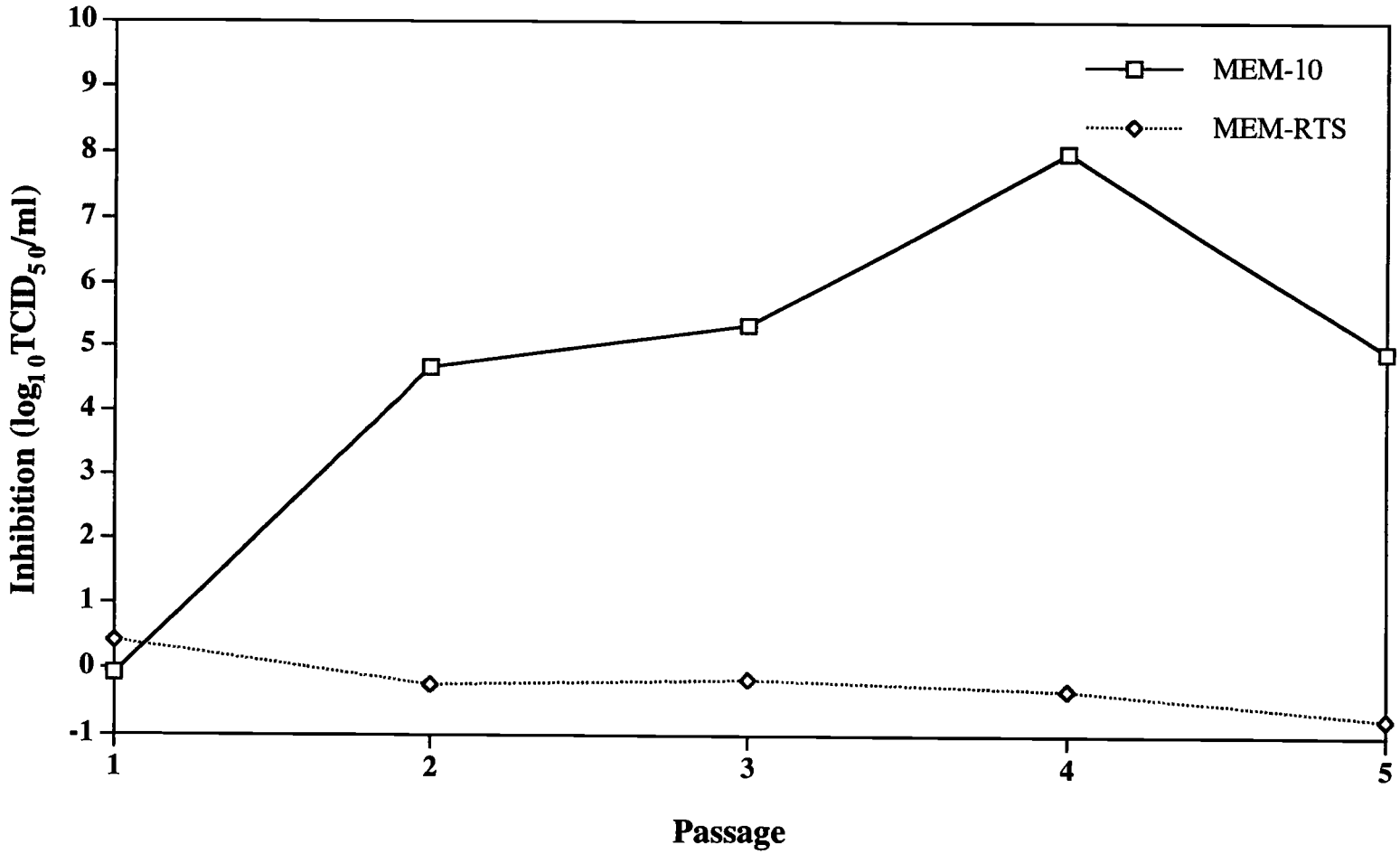
MEM-10
MEM-RTS
Inhibition ($log_{10}TCID_{50}$/ml)
10
9
8
7
6
5
4
3
2
1
0
-1
1
2
3
4
5
Passage

Figure 5.5

Figure 5.6 The effect of *in vitro* passage on sensitivity of IPNV 183-82 to RTS inhibitory activity *in vitro*. The virus was passaged five times with MEM-10 or MEM-RTS. Each of passaged virus with final concentration of $10^4 TCID_{50}$/ml in MEM-10 or MEM-RTS was infected to RTG-2 cells. Cells were incubated with either medium MEM-10 or MEM-RTS as appropriate during seven days. On the seventh day, virus titer from each medium was compared to determine RTS inhibition. The X-axis represents the passage number of IPNV 183-82 under each condition. The Y-axis represents the RTS sensitivity of each virus.

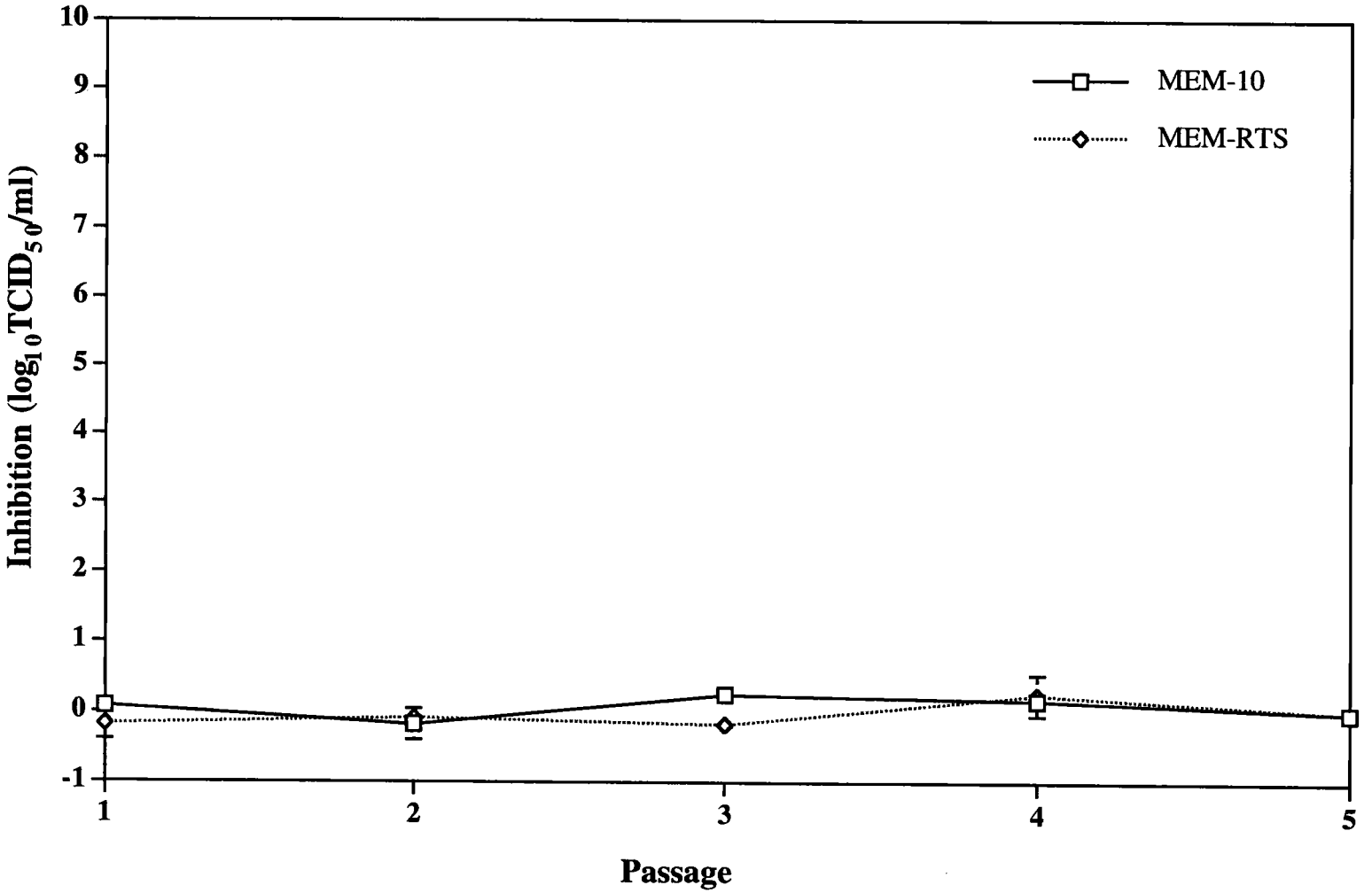
10
9
8
7
6
5
4
3
2
1
0
-1
Inhibition ($log_{10}TCID_{50}$/ml)
1
2
3
4
5
Passage
MEM-10
MEM-RTS

Figure 5.6

Table 5.2 Cross reaction in Immunodot Assay of Monoclonal Antibodies with three IPNV isolates, 90-11, crayfish, and 183-82 passaged five times in RTG-2 cell line with MEM-10 or MEM-RTS.

	Antibodies											
Virus Passages	AS1	W1	W2	W3	W4	W5	E1	E2	E3	E5	E6	E0[d]
MEM10-1[a]	+	-	-	+	-	+	+	-	-	+	+	-
MEM10-2	+	-	-	+	-	+	+	-	-	+	+	-
MEM10-3	+	-	-	+	-	+	+	-	-	+	+	-
MEM10-4	+	-	-	+	-	+	+	-	-	+	+	-
MEM10-5	+	-	-	+	-	+	+	-	-	+	+	-
MEM-RTS-1	+	-	-	+	-	+	+	-	-	+	+	-
MEM-RTS-2	+	-	-	+	-	+	+	-	-	+	+	-
MEM-RTS-3	+	-	-	+	-	+	+	-	-	+	+	-
MEM-RTS-4	+	-	-	+	-	+	+	-	-	+	+	-
MEM-RTS-5	+	-	-	+	-	+	+	-	-	+	+	-
Con-Buhl[b]	+	-	-	+	-	+	+	-	-	+	+	-
Con-W.B.[b]	+	+	+	+	+	+	+	-	-	+	+	-
Con-No Virus[c]	-	-	-	-	-	-	-	-	-	-	-	-

a: passage 1 passed in MEM-10

b: as positive controls, IPNV isolates Buhl and West Buxton (W.B.)

c: as a negative control, supernatant collected from uninfected cell monolayer

d: a negative monoclonal antibody which is not react with IPNV

DISCUSSION

Virulence

It has been suggested by many researchers that *in vitro* passage fewer than five times does not significantly alter virulence of IPNV, although virus loses its virulence to some degree through in vitro passage (Hill & Dixon 1977, Dorson et al. 1978). However, our results support the results of McAllister & Owens (1986) that significant decrease of viral virulence could have occurred even within five viral passages. The effects of RTS on preservation of virulence during multiple passages remained in question. Hill & Dixon (1977) tested Ab subtype strains and suggested that high virulent strain can preserve its virulence during mutiple passages in the presence of RTS and two of three low virulent strains can become high virulent if passaged with RTS. McAllister & Owens (1986), however, reported that RTS did not help to sustain virulence of the virus, VR-299 subtype isolate, tested. Ögut (1996) also found the variable effect of RTS on virulence, even within one subtype of IPNV (Buhl subtype). We also found variable effects of RTS to sustain virulence during five times of viral passages within the same subtype (Buhl subtype). Even though Hill & Dixon (1977), and Hill (1982) reported that Ab serotype low virulent IPNV isolate can become high virulent if passaged with RTS, we could not find any increased virulence when IPNV isolates were passaged with RTS. Overall, it was found that alterations in virulence with passage were isolate dependent. For example, when we compared the virulence change between first and 5^{th} passage 90-11 retained virulence when passaged in MEM-RTS (Figure 5.1) similar to the results of Ögut (1996). In contrast, crayfish isolate had elevated virulence in passage with MEM-10 than in

MEM-RTS (Figure 5.2). Until passage 4, 90-11 virulence was similar in the presence or absence of RTS, but by passage 5, virus passaged in either MEM-10 or MEM-RTS caused significantly decreased mortality. In one instance (passage 5 in MEM-10) the mortality was only 3%; however, the titer of virus isolated from these fish was not significantly different (10^1 $TCID_{50}$/ml difference) from titers in fish which had died during IPN epizootics (Figure 5.1). IPNV 183-82 passage 2 and 3 in MEM-RTS showed higher mortality than those in MEM-10 but not at other passages (Figure 5.3). Some researchers tried to determine if viral passage with RTS helped sustain viral virulence. We found that RTS does not always help to sustain virulence. Even one more passage in normal medium (MEM-10), sometimes, could cause dramatic virulence change which is higher change than virulence change induced by mediums used such as MEM-10 or MEM-RTS. The significant change in virulence with one viral passage was reported by some researchers (Sonstegard & McDermott 1971; Hill 1982; Linda 1997; Maret 1997). Originally avirulent strain of IPNV caused high mortality in following experimental challenge (Sonstegard & McDermott 1971; Hill 1982). Thus, it does not mean that it is avirulent just because a virus was isolated from healthy fish (carrier fish) or it is virulent just because a virus was isolated from diseased fish.

Nicholson et al (unpublished data) obtained sequence data from three isolate passages that we used, 90-11, crayfish, 183-82. They found genetic variations in VP1, VP2, NS, and VP5 protein of IPNV. However, sequence change was not related to virulence. Even same sequence results were obtained from the highest (passage 2 in MEM-RTS) and lowest passage virlence (passage 2 in fish, unpublished data) of 183-82.

If genetic change is not main reason of the virulence change by viral passage, it is question if isolate has its own characteristics on virulence.

In vitro virus sensitivity to RTS

The change of virus sensitivity to RTS was unpredictable. Ögut(1996) reported that in terms of serum inhibition, 23 from 29 Buhl subtype isolates proved to be unaffected by the presence of RTS and especially, 90-11 was resistant to RTS. However, we found that 90-11, originally RTS resistant, became RTS sensitive when it was passaged five times in RTG-2 with MEM-RTS (Figure 5.4). Ögut(1996) reported that crayfish had low level of RTS sensitivity (10^{-2}/ml $TCID_{50}$ reduction). We, however, found RTS sensitivity of this isolate was altered during viral passage (Figure 5.5). When the virus was passaged in MEM-10, it became RTS sensitive while the isolate passaged in MEM-RTS kept their resistance. Overall our results indicate that RTS sensitivity or resistance is not permanent but changeable, and viral passage in the presence of RTS could induce the development of an RTS sensitive variant or an RTS resistant variant.

In vitro virus sensitivity to RTS and its relationship to virulence

In conclusion, sensitivity of three IPNV to RTS did not show consistent relationship with viral virulence. Thus we suggest that RTS susceptibility *in vitro* is not a good prediction of viral virulence in brook trout. There are three possible reasons for this

lack of relationship between RTS sensitivity and virulence in brook trout. First, brook trout used in virulence test did not have enough 6S inhibitor, thus RTS sensitivity pattern *in vitro* was not linked to the virulence. We already found significant difference in the amount of 6S inhibitor between rainbow trout serum and brook trout serum (BTS) (Park unpublished data). Thus we obtained no consistent relationship from brook trout. Second, many defense molecules are involved in the viral defense of the host. Thus, many factors might be involved in virulence test. Thus, it was likely that the results of virus sensitivity to RTS *in vitro* did not correlate with virulence tests *in vivo* because other factors superceded the *in vitro* results. Third, the virus itself could have virulence factors which are more critical to virulence than virus sensitivity to RTS. The data from Nicholson's lab (unpublished data) showed no genetic relationship between high and low mortality or high and low sensitivity to RTS from the passages of three isolates, IPNV 90-11, IPNV crayfish, and IPNV 183-82. There is, overall, no relationship between sensitivity to RTS and genetic information. A recent publication (Hjalmarsson et al. 1999) reported evidence of the glycosylation of the capsid protein VP2 of IPNV, even though these results are at variance with other studies (Dobos 1995). If the difference in mortality and RTS sensitivity was not a result of genomic composition, the importance of phenotypic characteristics of the IPNV may still explain the relationship virulence and sensitivity to RTS. In another series of experiments, we found RTS sensitivity was changed by cell culture conditions (Park unpublished data). RTS sensitivity was dependent on cell concentration of host cells and was changed by cell type in which virus was replicated. If RTS resistance of IPNV is phenotypic characteristic, the ratio between resistant virus and

sensitive virus to RTS could effect on much part of the relationship between sensitivity to RTS and virulence.

Enzyme immunodot assay

We found identical monoclonal reactions with both RTS sensitive and RTS resistant IPNV strains. Some researchers (Kelly & Nielsen 1985, Ögut 1995) tested many serotype isolates to determine if sensitivity to RTS was related with IPNV serotypes. They found no such relationship. Thus we could suggest, based on our results and other publised information, that the change in RTS susceptibility during viral passage *in vitro* and *in vivo* is not related with epitope binding of RTS or epitope alteration. If 6S inhibitor directly binds to virus, then binding site of virus is not related with epitope region but one of capsid region which is commonly present on IPNV.

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CHAPTER 6

CLONES OF IPNV-JASPER ARE HETEROGENEOUS WITH RESPECT TO INACTIVATION BY NORMAL RAINBOW TROUT SERUM, SEROTYPE, AND GENE SEQUENCE

Kyoung Chul Park and Paul W. Reno

ABSTRACT

In order to determine if the infectious pancreatic necrosis virus isolate IPNV-Jasper (Ja) is homogeneous or heterogeneous with respect to inactivation by normal rainbow trout serum (RTS), 50 clones were tested for sensitivity to RTS. The initial isolate was very sensitive to RTS, losing up to 10^8 $TCID_{50}$/ml with a 1:100 dilution of RTS. The sensitivity of the clones ranged from highly sensitive to completely resistant (0-10^8 $TCID_{50}$/ml reduction). Eight percent of clones (4/50) were very sensitive to RTS (Ja-S) and eighty four percent of clones (42/50) showed a mid-range of sensitivity to RTS. The final eight percent of clones (4/50) were resistant to RTS (Ja-R). Enzyme immunodot assay revealed that Ja-S clones showed a monoclonal reaction typical to the parents, Ja; however, Ja-R clones differed by several epitopes from the parental strain. Analysis of two strains revealed that Ja-S and Ja-R had significant differences in their nucleic acid sequences for the capsid protein VP2. These two strains shared 80.7% and 86% identity in nucleic acid and in amino acid sequences, respectively. Ja-S had 99.7% and 91.0% identity in nucleic acid sequences and 99.5% and 95.9% in amino acid sequences with Ja and Jasper-Dobos (Ja-D), respectively, while Ja-R showed 80.6% and 79.9% identity in nucleic acid sequences and 86.5 and 87.1% in amino acid sequences with Ja and Ja-D, respectively. In conclusion, Ja population grown in the absence of RTS was heterogeneous in terms of RTS sensitivity, serotype, and dsRNA sequence.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) belongs to the family Birnaviridae (Dobos et al. 1979) and is an agent of an acute, contagious fish disease causing high mortality not only in juvenile salmonids but also in non-salmonid fishes (Reno 1999). IPNV has two segments of double-stranded RNA: segments A ($2.5x10^6$ Da) and B ($2.3x10^6$ Da). Segment B encodes the RNA-dependent RNA polymerase VP1 (94 kDa) while segment A contains two open reading frames. The short one encodes a 17-kDa polypeptide identified only in infected cells but not in purified cells (Duncan et al. 1991, Magyar and Dobos 1994). The long one encodes a 106-kDa polyprotein which is cotranslationally cleaved by a viral protease (NS or VP4, 29 kDa) into pVP2 (62 kDa) and VP3 (31 kDa) (MacDonald and Dobos 1981, Mertens and Dobos 1982, Duncan et al. 1987). VP3 was thought to be an internal protein of the virus (Dobos and Rowe 1977) but at least a portion of VP3 is exposed on the surface since it reacts with a number of monoclonal antibodies (Caswell-Reno et al. 1989). VP3 is genomically stable (Bruslind in press, Nicholson unpublished data). The pVP2 is further processed, during viral maturation, into VP2 which is major external protein and responsible for the reaction of type-specific neutalizing monoclonal antibodies (Dobos et al. 1977, Nicholson 1993). A universal, group-specific epitope has been reported to be located near the amino terminus of VP2, whereas the polypeptide responsible for a serotype-specific epitope has been mapped in the middle of the polypeptide (Dobos 1995). Heppell et al. (1995) reported that the central region of VP2 showed more variable deduced amino acid sequences than

its extremities. Thus, the focus has been on VP2 rather than VP3 in studies of the virulence of IPNV.

It has been reported that an anti-IPNV inhibitor, called "6S inhibitor", is present in rainbow trout serum (RTS) unexposed to IPNV (Vestergard-Jörgensen 1973, Dorson & de Kinkelin 1974). The inhibitor ("6S") is thought to be neither antibody nor interferon because the molecule has a sedimentation coefficient of approximately 6S by ultracentrifugation and, thus, is different from IgM-fish antibody which has a 14-16S sedimentation coefficient (Dorson & de Kinkelin 1974). It is not interferon because we also found (Park unpublished data) that the inhibitor has a molecular weight of approximately 150 kDa. IPNV is directly affected by the inhibitor before viral attachment (Kelly & Nielson 1985, Park unpublished data) but cells were not protected if they were pretreated with RTS. Not all virus isolates tested were inhibited by RTS and the inhibition was not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Okamoto et al. 1983, Ögut 1995). Cell culture-adapted virus strains were more susceptible to RTS than were wild type virus (Dorson & de Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978). It has been also reported by some researchers that RTS sensitivity was correlated with virulence of IPNV and was changed by cell passage (Hill and Dixon 1977, Hill BJ 1982, Ögut 1995). The development of RTS sensitivity of IPNV is dependent on the cell line in which the virus replicates. It has been reported that IPNV sensitivity to RTS was developed sooner with multiple viral passages in a cyprinid cell line (EPC) than in the RTG-2 or BF cell lines (Hill & Dixon 1977) and in RTG-2 cell line than CHSE-214 cell line (Park unpublished data).

In our previous experiments, we found much variation in the sensitivity of IPNV to RTS inhibitory activity even though we used the same RTS source, the same stock of virus, and the same host cell condition (tightly confluent). In some cases, the virus was totally resistant to RTS. In other experiments (Park unpublished data) using a member of the A_1 serotype (IPNV-Buhl), we found that sensitivity was altered after five passages (Park unpublished data). Sequencing of the sensitive and resistant variants revealed genetic differences in VP2 of sensitive and resistant variants, but not in VP3. Therefore, we questioned whether the virus population was heterogeneous in terms of RTS sensitivity. At present, no information is available about whether virus clones have different RTS sensitivity and different genetic information. Therefore, in this work, we first cloned an RTS sensitive strain of IPNV (Ja-S) and tested the progeny for RTS sensitivity. Second, the epitope pattern on VP2 and VP3 of clones with two different sensitivities was determined. Third, the cDNA sequences of VP2 region of two strains of IPNV-Jasper, Ja-S or Ja-R, were compared.

MATERIALS AND METHODS

Virus

The IPNV-Jasper isolate (ATCC VR-1325) used in this study belongs to serotype A_9 (Hill & Way 1995). This virus was isolated from diseased brook trout (*Salvelinus fontinalis*) at the Malgne River Hatchery, Alberta, Canada (Yamamoto 1974) and was

kindly provided by Dr. B. Nicholson, University of Maine, Orono, ME, U.S.A. and originally donated by Barry Hill (DAFF, Waymouth, U.K.). The virus has been passaged for 20 years in the laboratory. The virus was passaged two times through brook trout fry by immersion infection in 10^4TCID$_{50}$/ml water for 5h at 14°C and once in CHSE-214 cells (Lannan et al. 1984) prior to use in these experiments.

Isolation of clones sensitive or resistant to RTS

IPNV-Jasper (10^7TCID$_{50}$/ml) was serially diluted to 10^1TCID$_{50}$/ml and 0.1 ml of the final dilution was inoculated into 96 wells of each of 15 replicate 96 well plates. Fifty virus clones were isolated from 96 well plates showing CPE in fewer than 5 wells. The virus contained in these wells had a high probability of arising from a single virus. The isolated clones were tested for sensitivity to RTS *in vitro* as described below. Aliquots of isolated clones were kept in liquid nitrogen for later serotyping and sequencing.

***In vitro* virus sensitivity to RTS**

The RTG-2 cells (Wolf and Quimby 1962) were propagated in 24 well microtiter plates as described by Caswell-Reno et al (1989). Two wells were prepared for each clone; one well was for MEM-RTS and the other one was for MEM-10. In this experiment, three types of media were used: Eagle's Minimum Essential Medium (MEM) without serum=MEM-0; MEM+10% fetal bovine serum=MEM-10; MEM+10% fetal

bovine serum+1%RTS=MEM-RTS. Virus was diluted with MEM-10 or MEM-RTS to give a final virus concentration of $10^4 TCID_{50}$/ml. One hundred microliter of diluted virus were added onto a tightly confluent monolayer in each well and incubated at room temperature for two hours. After the incubation time, the inoculated monolayers were washed three times with MEM-0 and then 1ml of either MEM-10 or MEM-RTS was added. Uninoculated controls were treated with either medium, MEM-10 or MEM-RTS, as appropriate. Cells were incubated at 18°C for seven days in an incubator to which 5% CO_2 was supplied. The level of cytopathic effect (CPE) was monitored daily and scaled from 0 (no CPE) to 4 (complete CPE) on an ordinal scale. On the seventh day after exposure, cell culture supernatant from each well was harvested, pooled, and held in liquid nitrogen until they were titrated.

Virus titration

CHSE-214 cells (Lannan et al. 1984) were grown with MEM-10 in 96 well plates. The endpoint dilution method as described in Caswell-Reno et al. (1986) was used for virus titration. Virus samples were serially diluted 10-fold with MEM-0 and then 100μL of each of diluted virus suspension were added to each of four wells of a 96 well plate (Lannan et al. 1984). After incubation at 18°C for 7 days, wells showing CPE were counted to determine 50% tissue culture infectious dose ($TCID_{50}$/ml) (Spearman 1908).

Nucleic acid extraction

Viral RNA was extracted from two types of IPNV-Jasper clones, highly RTS sensitive (Ja-S) or RTS resistant (Ja-R), using TRIzol reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). Briefly, one hundred µl of virus ($10^7 TCID_{50}$/ml) were incubated with 1ml of TRIzol reagent for 5 min at room temperature. Two hundred µl of chloroform was added and incubated at room temperature for 3 min. After centrifugation at 11,500 x g for 15 min., six hundred µl of the top aqueous layer was mixed with 500 µl of isopropanol and 60 µl of 3M sodium acetate. Extracted RNA was centrifuged at 15,000 x g for 30 min. at 4°C then the resulting pellet was washed with 1ml of 75% ethanol. After one more centrifugation for 5 min, the pellet was air dried and the RNA resuspended in 20 µl Tris buffer (10mM Tris-Cl, pH8.5) and stored at –70°C.

Primers

Primers were designed based on published sequences of the cDNAs of genomic segment A of the Ja-D (Duncan & Dobos 1986). Primers for PCR and sequencing were constructed at the OSU Center for Gene Research. The primer sequences and location on the genome are shown in Table 6.1.

Table 6.1 Primers for RT-PCR and cDNA sequencing

Primer name	Orientation	Position*	Sequences (5' to 3')
F31	sense	31-53	TTGAGATCCATTATGCTTCCCGA
F37	sense	37-60	TCCATTATGCTTCCCGAGAATGGA
R148	antisense	148-125	TTCCTGAGTCTGAGACCTCTAAGT
F417	sense	417-437	CAGCTTGATGTCCCTGACAAC
R669	antisense	669-649	TGTTGGGGTCCCGGTTGCCAT
F735	sense	735-754	GCTAGAAGCCAAACCCGCCA
R1208	anrisense	1208-1182	AGGATCATCTTGGCATAGTTTAGGCCC
R1212	antisense	1212-1190	GGACAGGATCATCTTGGCATAGT
T7 promoter[a]	antisense	383-364	GTAATACGACTCACTATAGGG
M13 reverse[b]	sense	205-221	CAGGAAACAGCTATGACC

*The map positions of the primers are based on the sequence of segment A of the IPNV-Jasper (Duncan & Dobos 1986): the position 31 in this paper corresponds to position 151 of segment A of Jasper.
T7 promoter[a] and M13 reverse[b] indicate primer sets on the sequence of the cloning vector for VP2 of IPNV.

Reverse Transcription (RT)

Extracted RNA was diluted in RNase, DNase-free water to a concentration of 100 ng/µl RNA and heated at 95°C for 5 min, and placed on ice for 2 min. The following components were added to make a total of 50 µl reaction mixture: 2ul of diluted viral RNA (100 ng/µl), 2 µl (100 pmol) 3' primer, 2 µl (100 pmol) 5' primer, 6 µl M-MLV 5X reaction buffer (Promega, Madison, WI; 250mM Tris-HCl, pH 8.3 at 25°C, 375 mM KCl, 15mM $MgCl_2$, 50 mM DTT), 2 µl (2 mM each ATP, CTP, GTP, TTP) deoxynucleotides (dNTPs), 1.2 µl (40 units) RNasin (Promega), 2 µl (400 units) M-MLV reverse transcriptase (Promega), 32.8 µl water. The RT reaction was performed at 37°C for 1 hr. The reverse transcribed cDNA was stored at –70°C.

Polymerase Chain Reaction (PCR)

Ten µl of reverse transcribed cDNA (150 ng/µl) was heated at 95°C for 5 min, then cooled on ice, and briefly centrifuged at 10,000 x g. The following components were added to make a 50 µl reaction mixture: 5 µl 10X PCR buffer (GIBCOBRL), 1 µl deoxynucleotides (dNTPs) (10 mM each ATP, CTP, GTP, TTP), 2 µl 50 mM $MgCl_2$, 1 µl (50 pmol) 3' primer (R1212), 1 µl (50 pmol) 5' primer (F31), 2 µl reverse transcribed cDNA (300 ng), 0.5 µl (2.5 U) PLATINUM *Taq* DNA Polymerase (GIBCOBRL), 37.5 µl sterile distilled water. After mixing and brief centrifugation of the solution, one drop of mineral oil was overlaid on the surface. Amplification was performed in a

Programmable Thermal Controller (PCT-100, MJ Research, Inc.). After the solution was heated to 94°C for 4 min, 35 cycles were performed using the following program: 94°C – 1 min, 60°C – 1 min, 72 °C – 2 min. At the 35th cycle, a final extension time for 10 min was allowed and the sample was then held at 4°C until samples were retrieved.

Purification of PCR products

Twenty microliters of each of the RT-PCR products were analysed by electrophorosis through a 1% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml) in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA). The gel was electrophoresed at 75 V for 1.5 h and visualized with a UV light. The DNA band of expected size (1.2 kb) was cut and transferred to a microcentrifuge tube. DNA products from agarose gel slices were purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocol. Briefly, the gel slice was completely dissolved with Buffer QG and the dissolved sample was applied to the QIAquick column and centrifuged for 1 min. The column was washed with Buffer PE and centrifuged for 1 min. To elute DNA, 30 μl of Buffer EB (10 mM Tris-Cl, pH8.5) was added and then the column was centrifuged for 1 min. Eluted DNA was stored at –70°C until further use.

cDNA cloning

PCR products were cloned using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, a total of 6 µl Topo ligation reaction was prepared: 4 µl of purified DNA solution, 1 µl of salt (final concentration of 200mM NaCl, 10mM $MgCl_2$), 1 µl of Topo Vector. The solution was gently mixed and incubated for 5 min at room temperature and placed on ice. Immediately, the One Shot Transformation reaction followed. Two microliters of 0.5 M β-mercaptoethanol were added to TOP10 One Shot competent cells. After addition of 2 µl TOPO ligation reaction, One Shot cells were incubated on ice for 30 min. Cells were heat shocked for 30 seconds at 42 °C and incubated on ice for 4 min. Two hundred fifty microliters of SOC medium was added and incubated for 1 hr at 37°C. One hundred fifty microliters of transformed competent cells were spread on Luria-Bertani (LB) plates containing ampicillin. The LB plate was incubated overnight at 37°C. One white colony was inoculated into 2 ml of LB medium containing ampicillin and incubated at 37°C for 12 hr. A QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA) was used to purify plasmid DNA from cultured cells. One and a half ml of cultured cells was centrifuged at 13000 rpm for 30 sec. at 4°C. The dried pellet was resuspended in 250 µl of Buffer P1 containing Rnase A. After addition of 250 µl of Buffer P2, the tube was gently inverted 4-6 times. 350 µl of Buffer N3 was added and the tube was inverted 4-6 times. The tube was centrifuged for 10 min. Supernatant was transfered to QIAprep column and centrifuged 1 min. The column was additionally washed with 0.5 ml of Buffer PB. The

column was washed with 0.75 ml of Buffer PB and centrifuged again for 1 min. DNA was eluted in 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5).

Sequencing and phylogenetic analysis

Sequencing of the DNA was carried out at OSU Center for Gene Research using an automatic DNA sequencer (ABI PRISM Model 377). Sequences were determined by the chain terminator method of Sanger with fluoresence dye labelled dideoxynucleotides (Sanger et al. 1997). Each base in the sequence was determined at least three times in both directions using synthetic oligonucleotide primers (Table 6.1) designed from determined nucleotide sequences. Chromatograms (ABI prisms) of sequenced data were analyzed and assembled by the use of the MacVector software and Nucleotide discrepancies among sequence replicates were determined by majority consensus. The nucleotide sequence was translated into amino acids using MacVector software. The sequences of the sensitive strain and resistant strain to RTS were compared to the sequences of 11 aquatic birnaviruses available in GenBank or in the report of Ma (1996). The similarity and phylogenetic relationships, based on 385 amino acid sequences, were analyzed by the Clustal method with DNASTAR MEGALIGN program.

Protein structure analysis

Protein composition and antigenic index (James-Wolf method) of amino acid sequences were analyzed using the DNASTAR Protean program. Hydropathic plots for two strains were conducted by the Kyte-Doolittle method using the DNASTAR Protean program and more detailed information was obtained using SeqView program. Secondary structures of two strains were predicted after conversion of nucleotide sequences to amino acids, using the Foldit (light) program (Jesior et al 1994).

Enzyme immunodot assay

To determine if Ja-S and Ja-R clones of IPNV-Jasper have different epitopes, an enzyme immunodot assay was performed for the parent virus population, cloned Ja-S, and cloned Ja-R following the procedure of Caswell-Reno et al. (1989). Briefly nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA) was soaked in Tris-buffered saline (TBS), pH 7.5 for 30 min and then placed into a 96 well immunodot apparatus (Bio-Rad). One hundred µl of each virus (10^7 $TCID_{50}$/ml) was added into each horizontal row of 12 wells on the apparatus. After a 2 hr adsorption time, 100 µl of 3% bovine serum albumin (BSA) in TBS were added and allowed for one hour to block unbound sites on the membrane. After washing three times with 200 µl of TBS, 100 µl of each monoclonal antibodies (Mabs) were added to appropriate wells and incubated for one hour. After washing three times with TBS, 100 µl of a 1:1000 dilution of goat anti-mouse

IgG (conjugated with horseradish peroxidase) was added to each well and incubated for one hour. After washing three times, the membrane from was transferred to a tray and washed one more time with TBS. The membrane was incubated with substrate (1.7 ml of 0.3% 4-chloronaphthol in 100% methanol, 100µl of 3% hydrogen peroxide, and 8.2 ml of TBS). Reaction was stopped by washing three times with TBS when the background began to turn purple. Positive reactions were indicated by a purple color. As positive controls, six serotype isolates including IPNV-Ab and Canada 1, were used. Supernatant collected from uninfected CHSE-214 cell culture was used as a negative-control antigen.

RESULTS

In vitro virus sensitivity to RTS

In order to determine if the population of IPNV-Jasper is homogeneous or heterogeneous in terms of RTS sensitivity, 50 clones were isolated from a virus population which had been passaged twice through brook trout then once in CHSE-214 cells and tested for RTS sensitivity. The parent isolate (Ja) was extremely sensitive to RTS, losing $10^{8}TCID_{50}$/ml when treated with RTS. The RTS sensitivity of each clone ranged from highly sensitive to resistant (Figure 6.1). Eight percent of clones were very sensitive (Ja-S) as was the parent strain (Ja). They did not show CPE during 7 days incubation in the presence of RTS and were inhibited as high as $10^{-4.8}TCID_{50}$/ml

Figure 6.1 Distribution of sensitivity to RTS among 50 clones of RTS sensitive parental IPNV-Jasper. Each clone was tested for RTS sensitivity. Very sensitive clones had no CPE during 7 days incubation in the presence of RTS; mid-sensitive clones showed up to 75% destruction of cell monolayers, in which cell destruction in the presence of RTS was less than cell destruction in the absence of RTS; Resistant clones showed the same level of cell destruction, greater than 75%, in the presence or in the absence of RTS.

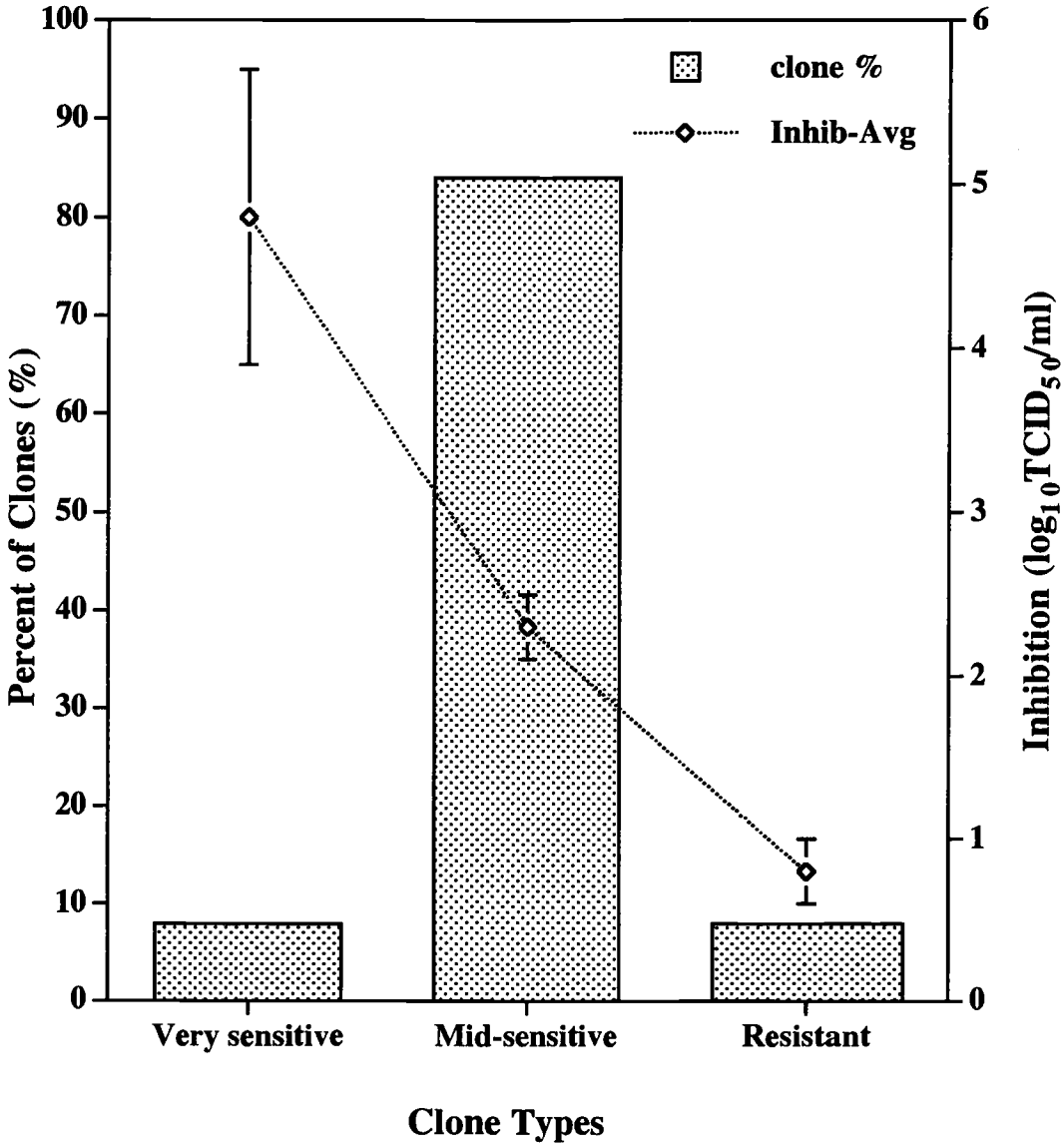
100
90
80
70
60
50
40
30
20
10
0
Percent of Clones (%)
6
5
4
3
2
1
0
Inhibition ($log_{10}TCID_{50}$/ml)
clone %
Inhib-Avg
Very sensitive
Mid-sensitive
Resistant
Clone Types

Figure 6.1

reduction. Eighty four percent of clones showed a mid-range of RTS sensitivity. CPE levels of these clones as measured on an ordinal scale of 0-3 in the presence of RTS were 1 to 2, while it was 3 in the absence of RTS. The mean inhibition level was $10^{-2.3}$ $TCID_{50}$/ml reduction. Eight percent of clones were RTS resistant (Ja-R). They were not significantly inhibited by RTS (mean $10^{-0.8}$TCID $_{50}$/ml reduction). The CPE-development with these clones was the same in the presence or absence of RTS (3 on our scale). CPE had developed by the second day postinfection.

Nucleotide sequences

The nucleotide sequences of Ja-S and Ja-R clones of IPNV were compared to determine if the difference in RTS sensitivity was related to specific genetic information. An 1182 bp fragment representing most of VP2 was sequenced and then compared with information on IPNV-Jasper published by Dobos (Ja-D) and IPNV-Jasper (ATCC VR-1325) (Ja) using the DNASTAR MegAlign program. The conserved initiation codon (ATG) of the large ORF published by Duncan and Dobos (1986) was defined as position 1 in the sequence. Thus, our sequence result was obtained from position 31 to position 1212 (Figure 6.2). Ja-S and Ja-R showed a low level of identity to each other (80.7%). The Ja-S was found to have high identity with Ja (99.7%); however, Ja-R showed only 80.6% identity with Ja. Compared to Ja-D, Ja-S showed a much higher identity (91.0%) than Ja-R (79.9%).

Figure 6.2 Multiple alignment of nucleotide sequences of an 1182 bp fragment representing most of the VP2 coding region in segment A of IPNV. The conserved initiation codon (ATG) of the large ORF was defined as position 1, based on the sequence of Jasper-Dobos (Duncan & Dobos 1986). Each isolate is abbreviated as follows: JA-D = Jasper isolate (Duncan & Dobos 1986); JA = Jasper-ATCC (Ma 1996); JA-S = Jasper isolate which is sensitive to RTS; JA-R = Jasper isolate which is resistant to RTS. Boxes surrounding nucleotides indicate differences from the majority sequence.

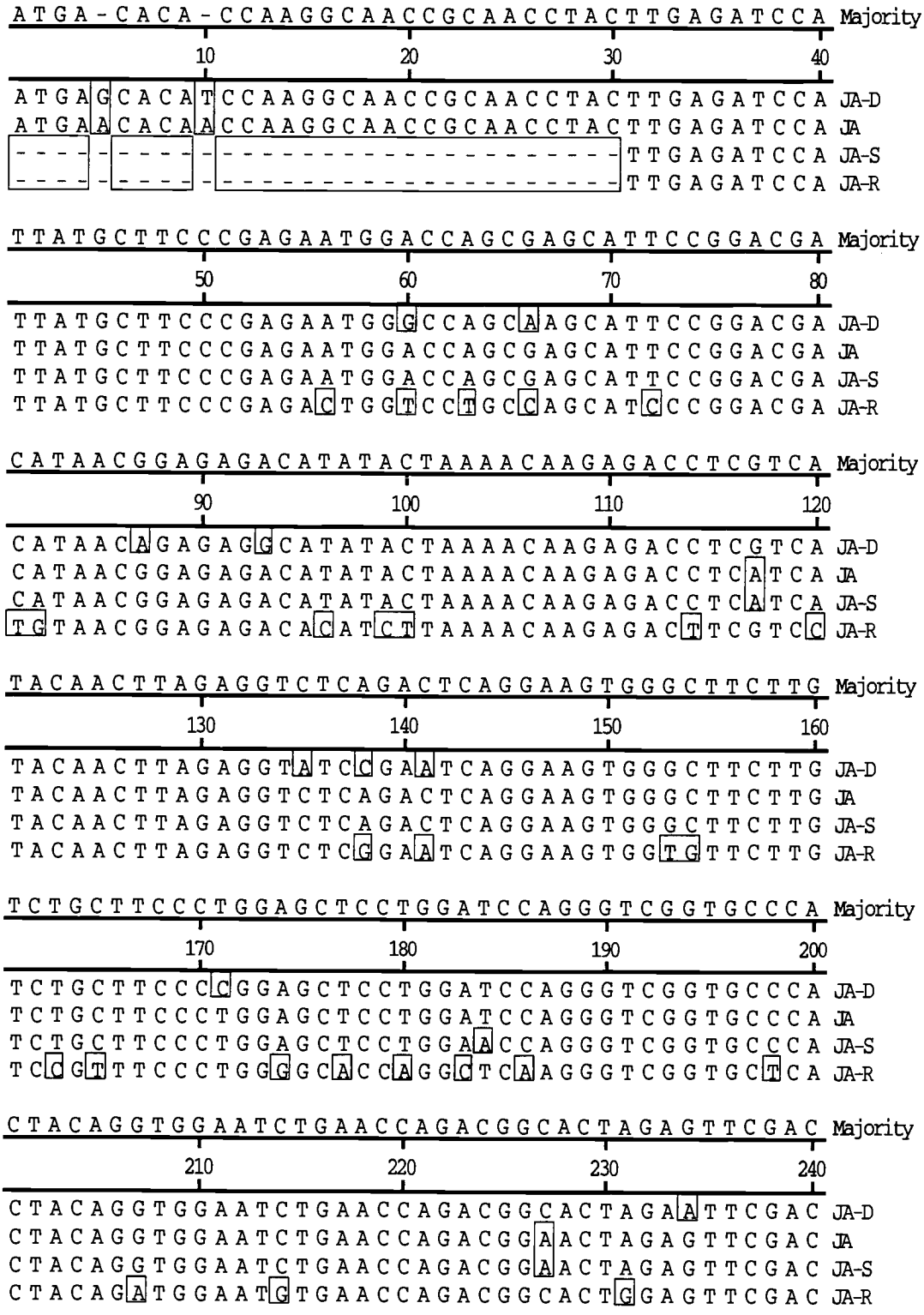
ATGA-CACA-CCAAGGCAACCGCAACCTACTTGAGATCCA Majority
10 20 30 40
ATGAGCACATCCAAGGCAACCGCAACCTACTTGAGATCCA JA-D
ATGAACACAACCAAGGCAACCGCAACCTACTTGAGATCCA JA
TTGAGATCCA JA-S
TTGAGATCCA JA-R
TTATGCTTCCCGAGAATGGACCAGCGAGCATTCCGGACGA Majority
50 60 70 80
TTATGCTTCCCGAGAATGGGCCAGCAAGCATTCCGGACGA JA-D
TTATGCTTCCCGAGAATGGACCAGCGAGCATTCCGGACGA JA
TTATGCTTCCCGAGAATGGACCAGCGAGCATTCCGGACGA JA-S
TTATGCTTCCCGAGACTGGTCCTGCCAGCATCCCGGACGA JA-R
CATAACGGAGAGACATATACTAAAACAAGAGACCTCGTCA Majority
90 100 110 120
CATAACAGAGAGGCATATACTAAAACAAGAGACCTCGTCA JA-D
CATAACGGAGAGACATATACTAAAACAAGAGACCTCATCA JA
CATAACGGAGAGACATATACTAAAACAAGAGACCTCATCA JA-S
TGTAACGGAGAGACACATCTTAAAACAAGAGACTTCGTCC JA-R
TACAACTTAGAGGTCTCAGACTCAGGAAGTGGGCTTCTTG Majority
130 140 150 160
TACAACTTAGAGGTATCCGAATCAGGAAGTGGGCTTCTTG JA-D
TACAACTTAGAGGTCTCAGACTCAGGAAGTGGGCTTCTTG JA
TACAACTTAGAGGTCTCAGACTCAGGAAGTGGGCTTCTTG JA-S
TACAACTTAGAGGTCTCGGAATCAGGAAGTGGTGTTCTTG JA-R
TCTGCTTCCCTGGAGCTCCTGGATCCAGGGTCGGTGCCCA Majority
170 180 190 200
TCTGCTTCCCCGGAGCTCCTGGATCCAGGGTCGGTGCCCA JA-D
TCTGCTTCCCTGGAGCTCCTGGATCCAGGGTCGGTGCCCA JA
TCTGCTTCCCTGGAGCTCCTGGAACCAGGGTCGGTGCCCA JA-S
TCCGTTCCCTGGGGCACCAGGCTCAAGGGTCGGTGCTCA JA-R
CTACAGGTGGAATCTGAACCAGACGGCACTAGAGTTCGAC Majority
210 220 230 240
CTACAGGTGGAATCTGAACCAGACGGCACTAGAATTCGAC JA-D
CTACAGGTGGAATCTGAACCAGACGGAACTAGAGTTCGAC JA
CTACAGGTGGAATCTGAACCAGACGGAACTAGAGTTCGAC JA-S
CTACAGATGGAATGTGAACCAGACGGCACTGGAGTTCGAC JA-R

Figure 6.2

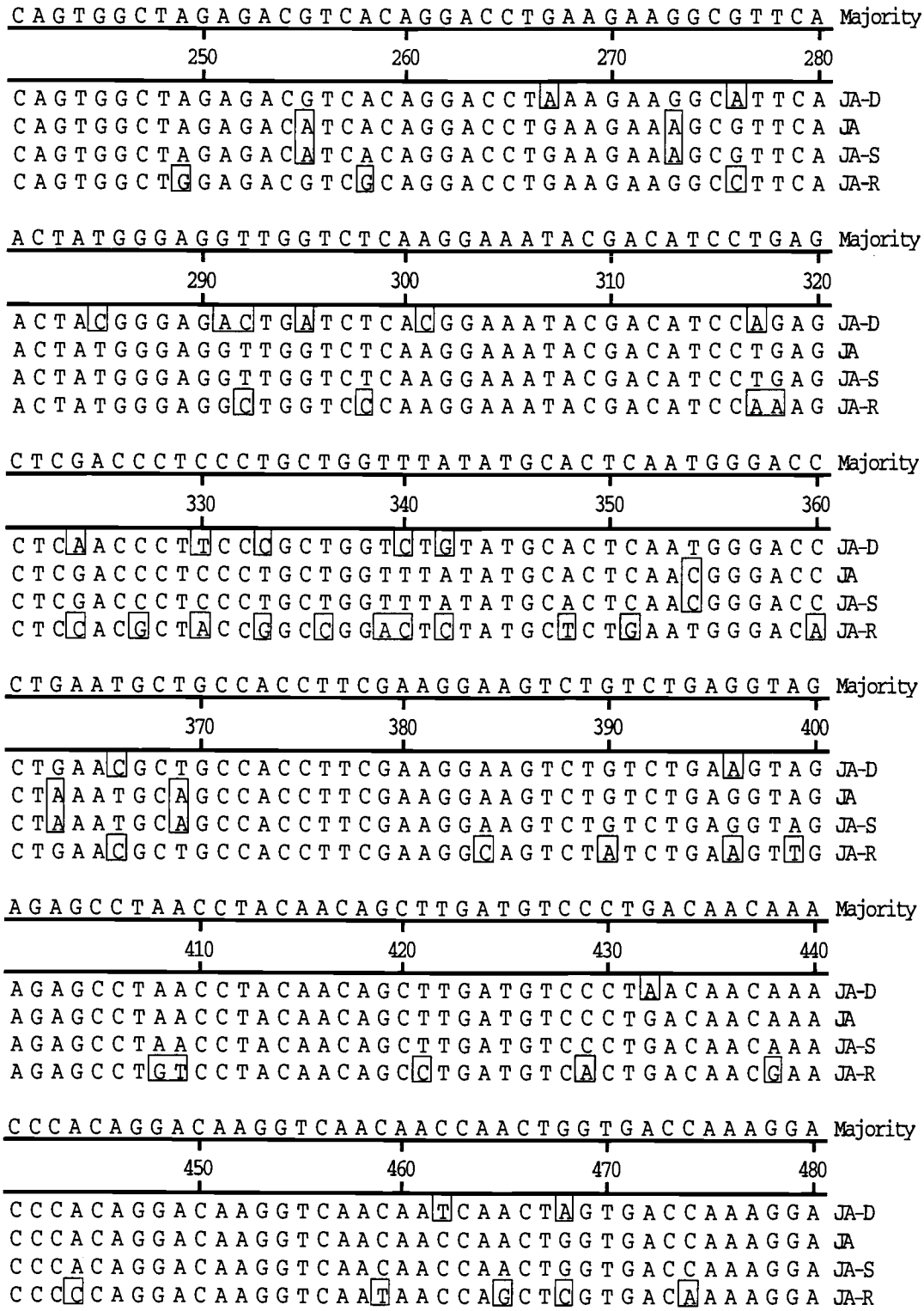

Figure 6.2 Continued

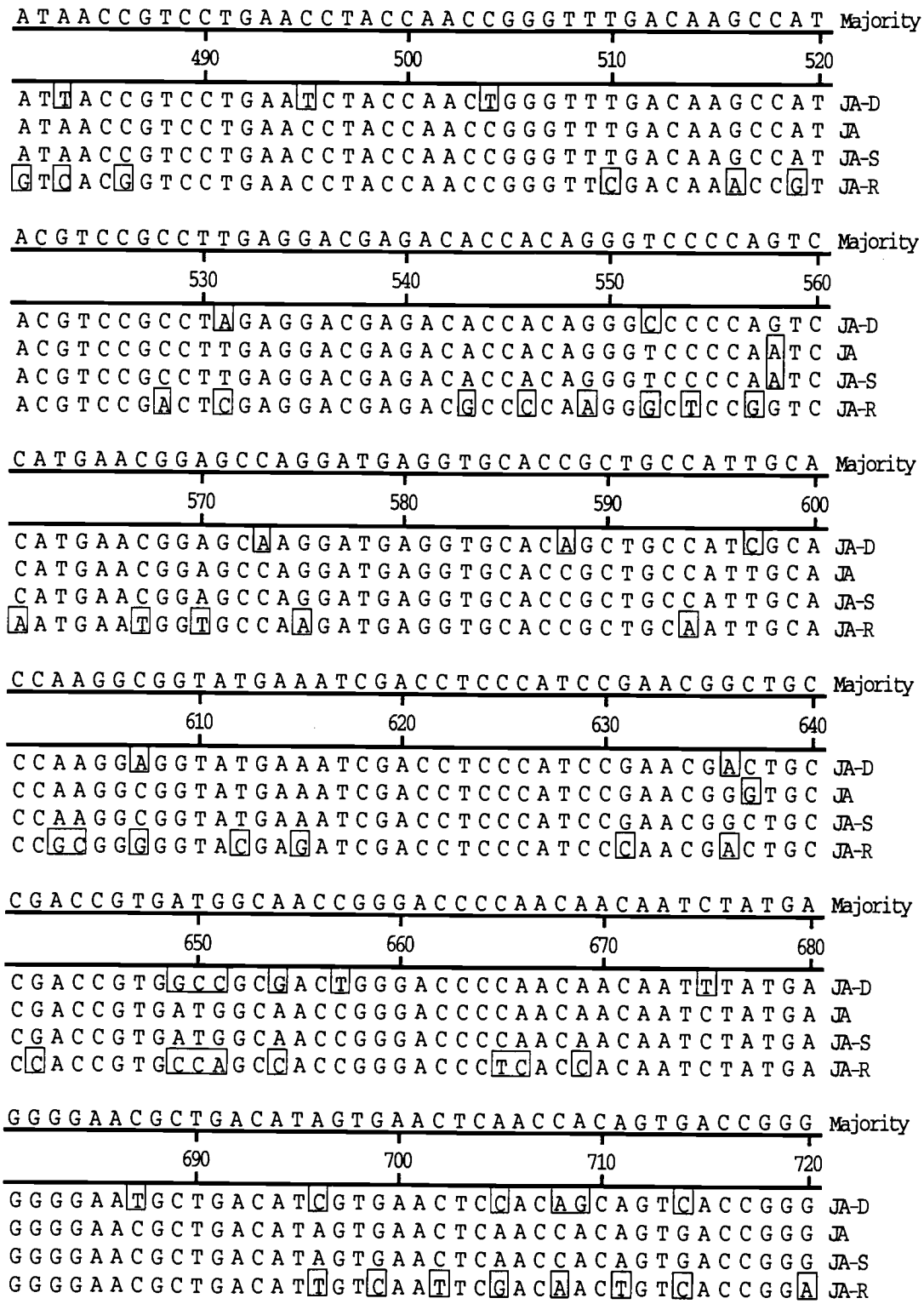

Figure 6.2 Continued

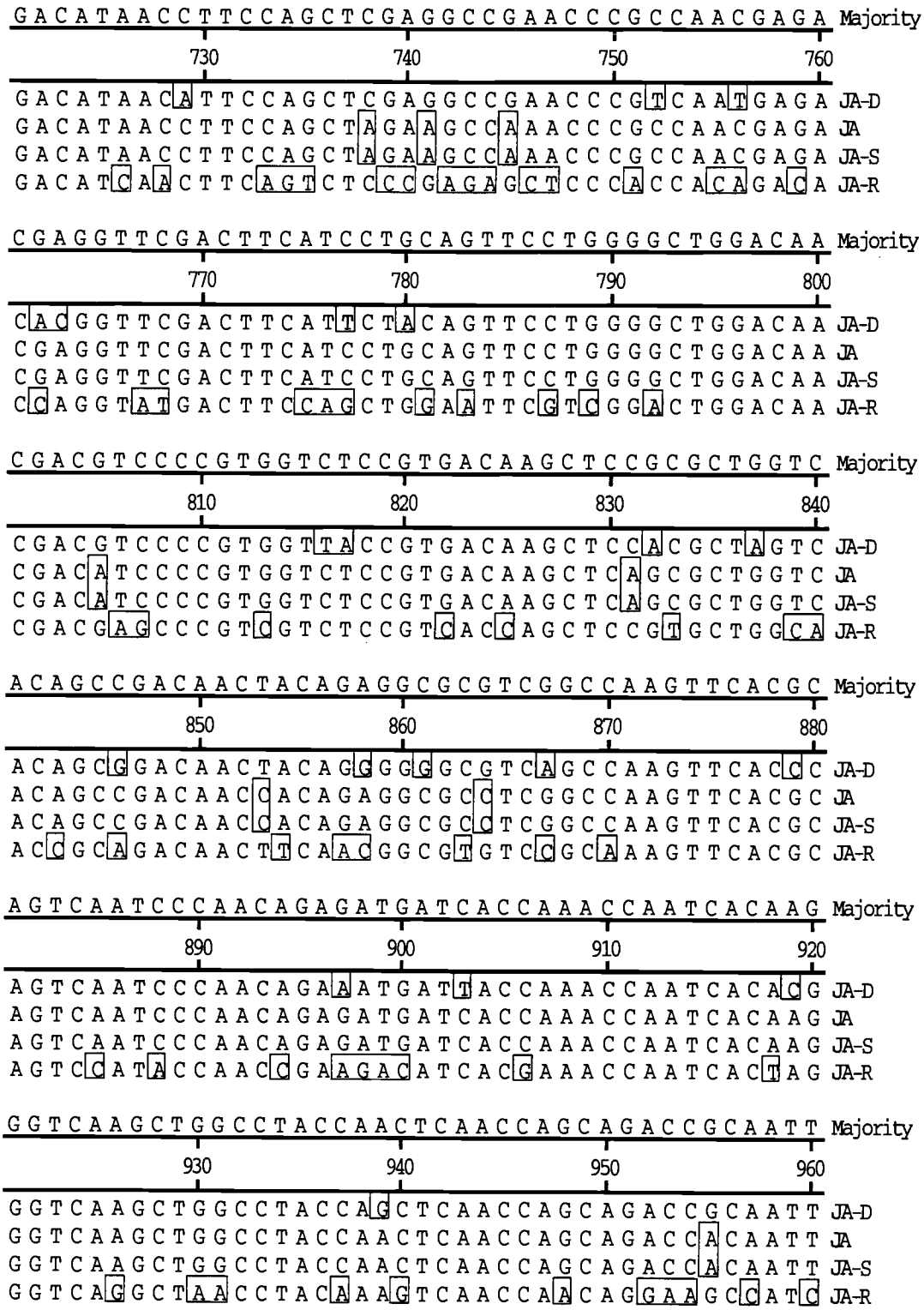

Figure 6.2 Continued

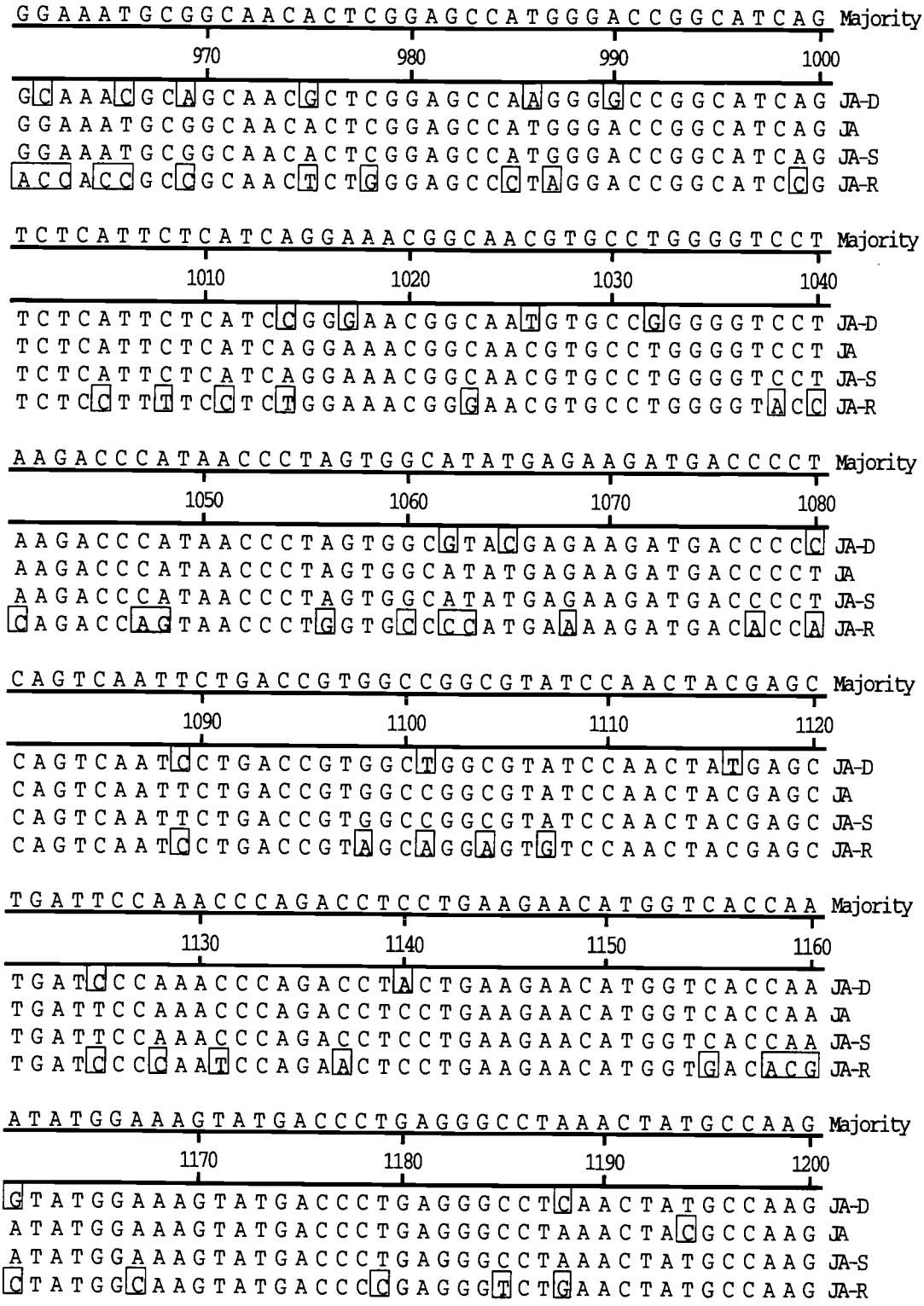

Figure 6.2 Continued

Figure 6.2 Continued

In percent divergence, Ja-S showed 0.3%, 9.6%, and 22.3% divergence from Ja, Ja-D, and Ja-R, respectively; however, RTS-resistant strain had 22.5%, 22.3%, and 23.5% divergence from Ja, Ja-S, and Ja-D.

Deduced amino acid sequences

Deduced amino acid sequences were obtained from translation of the 1182 bp nucleotide sequences using MacVector software. Translated amino acid sequences of the two strains were compared with published Ja-D and Ja using the DNASTAR MegAlign program (Figure 6.3). The comparison was obtained from amino acid positions 11 to 395. Although the amino acid sequences among Ja isolates were similar to those expected from the nucleotide sequences, some of the nucleotide discrepancies were silent and did not change the amino acid sequence. This resulted in higher similarity level in amino acid sequences than those of the nucleotide sequences. Ja-S and Ja-R were 86.5% identical. Ja-S, the main group in the RTS sensitive test, was found to be closely related to Ja (99.5%) and had an amino acid identity of 95.9% with Ja-D. Ja-R, however, showed only 86.5% identity with Ja and 87.1% with Ja-D.

Ja-S showed divergence of 0.5% with Ja, 4.2% with Ja-D, and 14.9% with Ja-R. The Ja-R sequence had divergence of 14.9% with Ja, 14.2% with Ja-D, and 14.9% with Ja-S.

On the other hand, the amino acid sequences of Ja-S and Ja-R were compared with published amino acid sequences of 11 aquatic birnaviruses showing high identity.

Based on the comparison of 385 amino acid residues, similarity (Table 6.2) and divergence matrices (Table 6.2), and a phylogenetic tree (Figure 6.4) were constructed. Ja-S had the highest identity with Ja (99.2%) and had high identity with DRT (96.4%), West Buxton (ATCC VR-877) (96.1%), Ja-D (95.8%), and VR-299 (ATCC VR-299) (95.8%). Ja-R was not grouped with those viruses, but had closer relation to Ab (ATCC VR-1319), Sp (ATCC VR-1318), Y-6, and N1. Ja-R showed a relatively low identity with other selected aquatic birnaviruses. The highest identity was found with IPNV-Ab (88.1%), followed by Sp (Tseng et al. 1996) (87.3%), Y-6 (87.3%), and N1 (87.0%); however, only 85.0% identity was found with Canada 2.

Protein structure analysis

Protein composition for two strains was analyzed using DNASTAR Protean program. The two strains had similar molecular weight: 41836 Da in Ja-S and of 41723 Da in Ja-R. Both strains had the same isoelectric point of 5.0. Ja-S showed 32.5% and 32.2% of polar residues and hydrophobic residues, respectively, while, Ja-R had 32.5% and 31.7%. Hydrophilic plots of the two strains were compared (Figure 6.6). Especially, there was a marked, significant difference in hydrophilic region found in Ja-S(S) and Ja-R(R): 63-69 (R), 73-75 (S), 257-259 (S), 275-278 (R), 339-343 (R). At those regions, only one strain had at least 3 consecutive hydrophilic residues, while the other strain did not show any hydrophilic residues. The difference between hydrophilic regions on the protein of the two strains were closely related to the estimated antigenic difference

Table 6.2 The percent similarity and divergence of the amino acid sequences of the 1155 bp cDNA fragment within the VP2 coding region of aquatic birnaviruses. Each virus is abbreviated as follows: Jas-D = Jasper-Dobos (Duncan & Dobos 1986); ATCC = Jasper-ATCC (Ma 1996); Jas-S = Jasper-RTS sensitive; Jas-R = Jasper-RTS resistant; Ab (Heppell e al. 1993); C-2 = Canada 2 (Heppell et al. 1993); DRT (Chung e al. 1994); N1 (Havastein et al. 1990); Sp* = Sp (Mason & Leong 1996); Sp** = Sp (Tseng et al. 1996); VR = VR-299 (Heppell et al 1993); WB = West Buxton (Yao & Vakharia 1998); Y-6 = (Suzuki 1998). The upper triangle indicates the percent similarity, while the lower triangle indicates the percent divergence.

Virus	Jas-D	ATCC	Jas-S	Jas-R	Ab	C-2	DRT	N1	Sp*	Sp**	VR	WB	Y-6
Jas-D	**	95.6	95.8	86.8	88.3	88.3	99.0	88.1	87.3	88.8	100.0	97.7	90.1
ATCC	4.6	**	99.2	86.0	86.8	88.1	96.1	86.8	86.0	87.5	95.6	95.8	89.4
Jas-S	4.3	0.8	**	86.2	87.0	88.3	96.4	87.0	86.2	87.8	95.8	96.1	89.6
Jas-R	14.6	15.6	15.3	**	88.1	85.7	86.8	87.0	85.7	87.3	86.8	86.2	87.3
Ab	12.7	14.6	14.3	13.0	**	87.5	88.1	90.6	88.6	90.1	88.3	88.6	89.9
C-2	12.7	13.0	12.7	15.9	13.7	**	89.1	89.6	87.5	89.1	88.3	88.3	88.1
DRT	1.0	4.0	3.7	14.6	13.0	11.8	**	88.3	87.5	89.1	99.0	97.1	90.1
N1	13.0	14.6	14.3	14.3	10.0	11.2	12.7	**	96.1	97.4	88.1	88.1	88.8
Sp*	14.0	15.6	15.3	15.9	12.4	13.7	13.7	4.0	**	97.7	87.3	87.0	87.3
Sp**	12.1	13.7	13.4	14.0	10.6	11.8	11.8	2.6	2.4	**	88.8	88.6	88.8
VR	0.0	4.6	4.3	14.6	12.7	12.7	1.0	13.0	14.0	12.1	**	97.9	90.1
WB	2.4	4.3	4.0	15.3	12.4	12.7	2.9	13.0	14.3	12.4	2.4	**	90.9
Y-6	10.6	11.5	11.2	14.0	10.9	13.0	10.6	12.1	14.0	12.1	10.6	9.7	**

Figure 6.3 Multiple alignment of deduced amino acid sequences of the 1182 bp cDNA fragment representing most of the VP2 protein of IPNV. Boxes indicate differences with the consensus. Each isolates indicate as follows: JA-D = Jasper isolate (Duncan & Dobos 1986); JA = Jasper-ATCC (Ma 1996); JA-S = Jasper isolate which is sensitive to RTS; JA-R = Jasper isolate which is resistant to RTS.

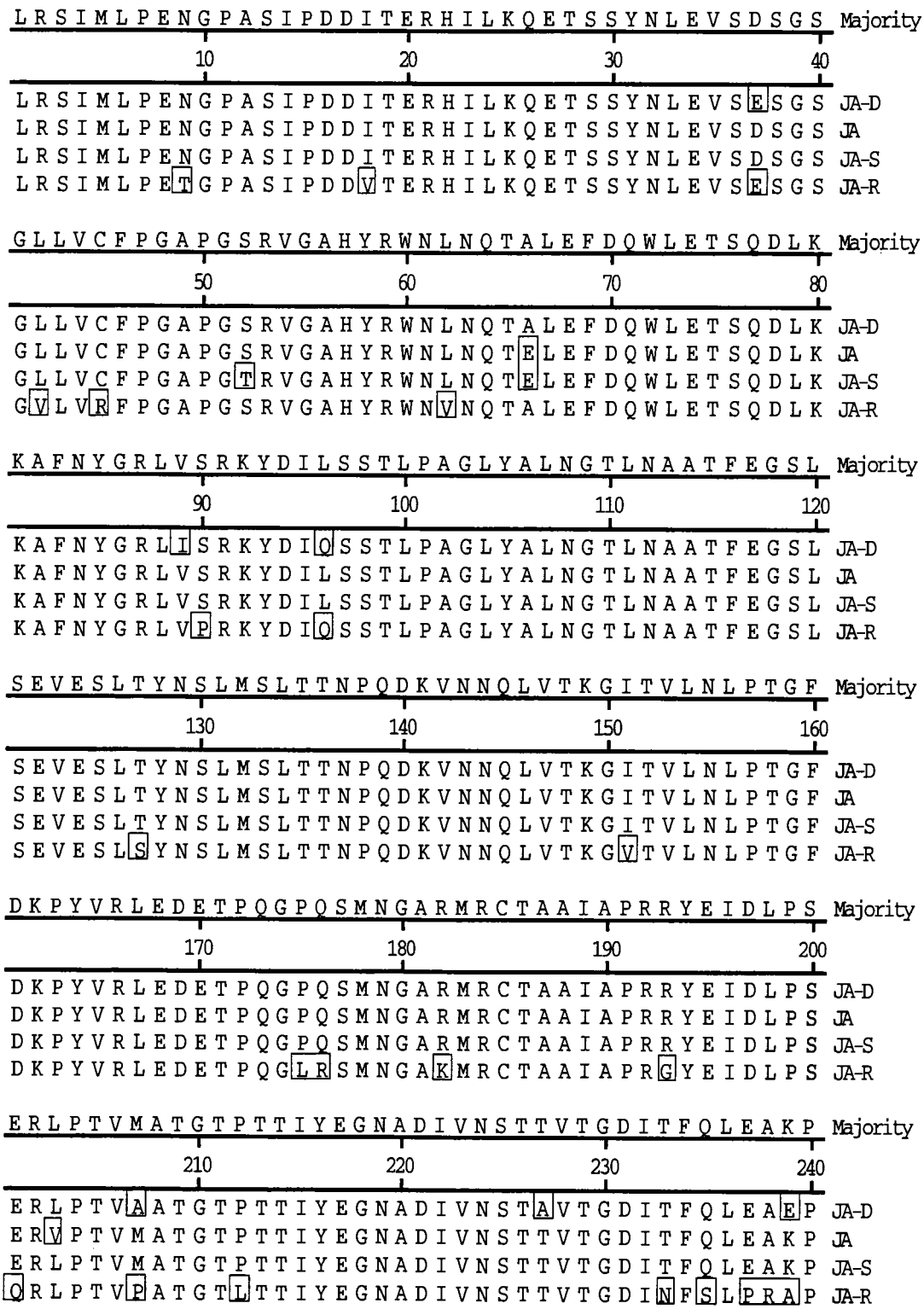
LRSIMLPENGPASIPDDITERHILKQETSSYNLEVSDSGS Majority
10 20 30 40
LRSIMLPENGPASIPDDITERHILKQETSSYNLEVSESGS JA-D
LRSIMLPENGPASIPDDITERHILKQETSSYNLEVSDSGS JA
LRSIMLPENGPASIPDDITERHILKQETSSYNLEVSDSGS JA-S
LRSIMLPETGPASIPDDVTERHILKQETSSYNLEVSESGS JA-R
GLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLK Majority
50 60 70 80
GLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLK JA-D
GLLVCFPGAPGSRVGAHYRWNLNQTELEFDQWLETSQDLK JA
GLLVCFPGAPGTRVGAHYRWNLNQTELEFDQWLETSQDLK JA-S
GVLVRFPGAPGSRVGAHYRWNVNQTALEFDQWLETSQDLK JA-R
KAFNYGRLVSRKYDILSSTLPAGLYALNGTLNAATFEGSL Majority
90 100 110 120
KAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATFEGSL JA-D
KAFNYGRLVSRKYDILSSTLPAGLYALNGTLNAATFEGSL JA
KAFNYGRLVSRKYDILSSTLPAGLYALNGTLNAATFEGSL JA-S
KAFNYGRLVPRKYDIQSSTLPAGLYALNGTLNAATFEGSL JA-R
SEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGF Majority
130 140 150 160
SEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGF JA-D
SEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGF JA
SEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGF JA-S
SEVESLSYNSLMSLTTNPQDKVNNQLVTKGVTVLNLPTGF JA-R
DKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEIDLPS Majority
170 180 190 200
DKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEIDLPS JA-D
DKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEIDLPS JA
DKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEIDLPS JA-S
DKPYVRLEDETPQGLRSMNGAKMRCTAAIAPRGYEIDLPS JA-R
ERLPTVMATGTPTTIYEGNADIVNSTTVTGDITFQLEAKP Majority
210 220 230 240
ERLPTVAATGTPTTIYEGNADIVNSTAVTGDITFQLEAEP JA-D
ERVPTVMATGTPTTIYEGNADIVNSTTVTGDITFQLEAKP JA
ERLPTVMATGTPTTIYEGNADIVNSTTVTGDITFQLEAKP JA-S
QRLPTVPATGTLTTIYEGNADIVNSTTVTGDINFSLPRAP JA-R

Figure 6.3

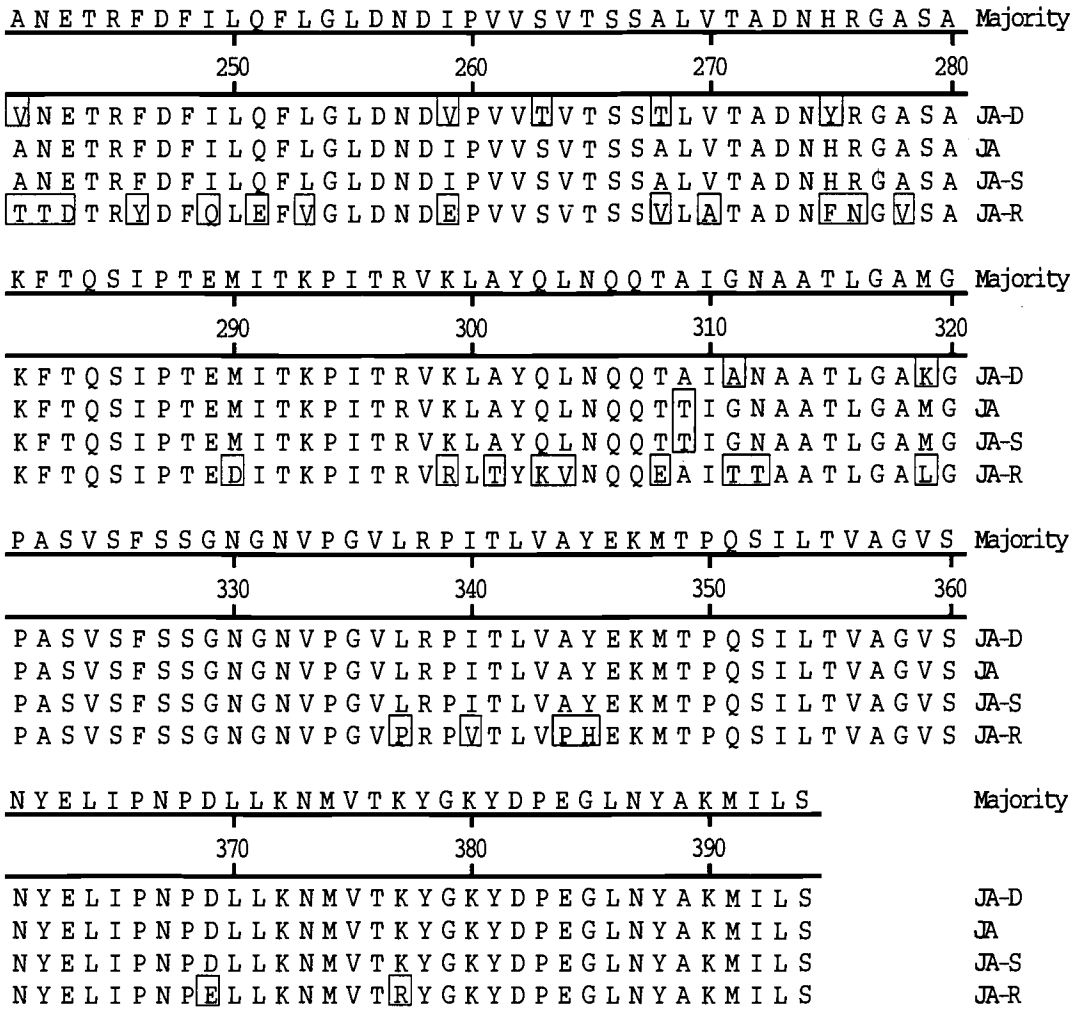

Figure 6.3 Continued

between the two clones (Figure 6.6). Significant antigenic differences were found especially at these regions: 60-70, 125-130, 230-340.

The predicted secondary structures of proteins translated from nucleotide sequences of Ja-S and Ja-R strain were predicted using Foldit (light) program. As shown in Figure 6.5, Some amino acid substitutions could change potential secondary structure of protein. Amino acid change between Ja-S and Ja-R at positions 99 (Ser to Pro), 175 (Pro to Leu), 207 (Met to Pro), 212 (Pro to Leu), 237 (Glu to Pro), 337 (Leu to Pro), and 344 (Ala to Pro) affected the predicted secondary structure of Ja-R.

Enzyme immunodot assay

The difference of monoclonal antibody reaction profile was determined from parent Ja, Ja-S and Ja-R. As shown in Table 6.3, parent Ja and progeny Ja-S showed same epitope pattern, such as in typical monoclonal antibody reaction of Ja, showing positive reactions with AS-1, W-1, W-2, W-3, W-4, W-5, E-1 and E5. Ja-R, however, had significantly different monoclonal reaction, in that they reacted with only AS-1, E-1, E-5, and E-6. Thus, this strain did not react with W-1, W-2, W-3, W-4, and W-5 Mabs but also reacted with one more Mab E-6.

Figure 6.4 Phylogenetic tree of selected aquatic birnaviruses based on deduced amino acid sequences of an 1155 bp cDNA fragment encoding most of VP2. Each virus is abbreviated as follows: Ja-D = Jasper-Dobos (Duncan & Dobos 1986); Ja = Jasper-ATCC (Ma 1996); Ja-S = Jasper-sensitive strain to RTS; Ja-R = Jasper-resistant strain to RTS; Ab (Heppell e al. 1993); C-2 = Canada 2 (Heppell et al. 1993); DRT (Chung e al. 1994); N1 (Havastein et al. 1990); Sp* = Sp (Mason & Leong 1996); Sp** = Sp (Tseng et al. 1996); VR = VR-299 (Heppell et al 1993); WB = West Buxton (Yao & Vakharia 1998); Y-6 = (Suzuki 1998).

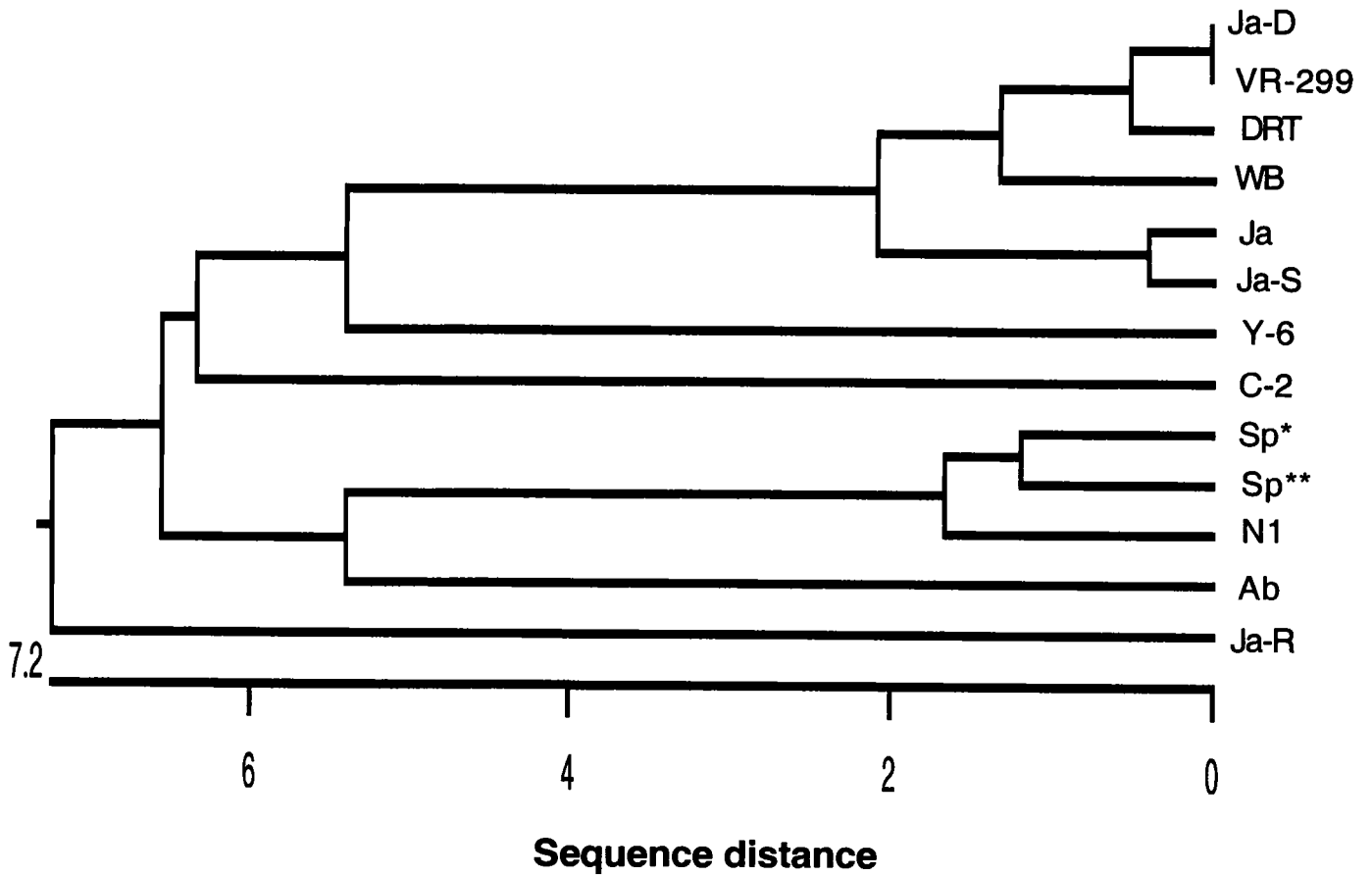
Ja-D
VR-299
DRT
WB
Ja
Ja-S
Y-6
C-2
Sp*
Sp**
N1
Ab
Ja-R
7.2
6
4
2
0
Sequence distance

Figure 6.4

Figure 6.5 Predicted protein secondary structure of the VP2 protein from deduced amino acid sequences of the 1182 bp cDNA sequences. A show the secondary structure of IPNV-Jasper which is sensitive to RTS and B indicates resistant clone to RTS. Both are oriented identically on their X, Y, Z axes.

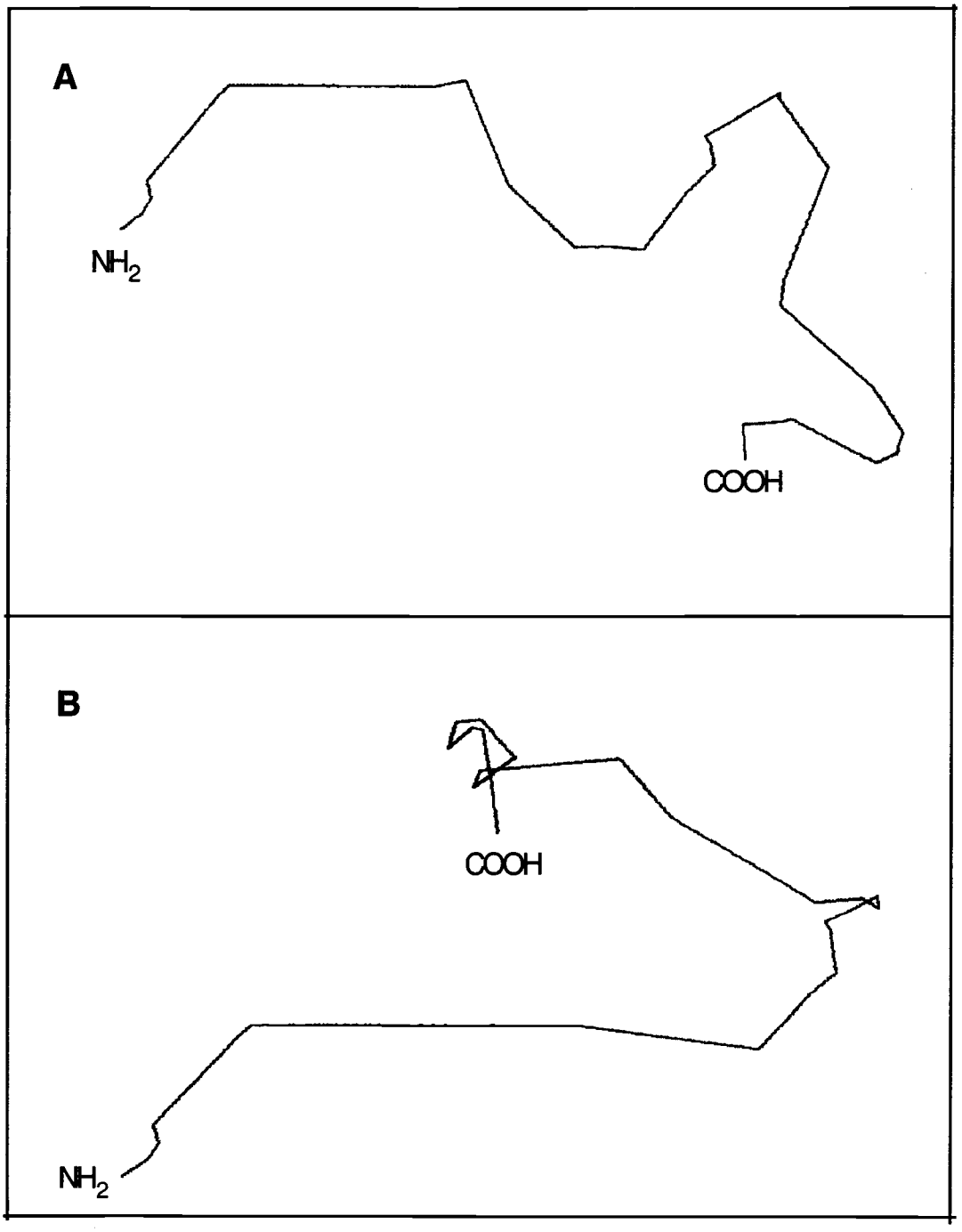

Figure 6.5

Figure 6.6 Hydrophilicity plots and antigenic index of amino acid sequences of VP2 from two IPNV-Jasper strains. Upper box A is for Jasper-RTS sensitive strain; box B is depicts the RTS resistant strain. Hydrophilicity plots were determined by the Kyte-Doolittle method. Antigenic indexes were determined by the James-Wolf method. The ruler represents the amino acid position. The lines under the antigenic index show the major antigenic differences between RTS sensitive strain and RTS resistant strain.

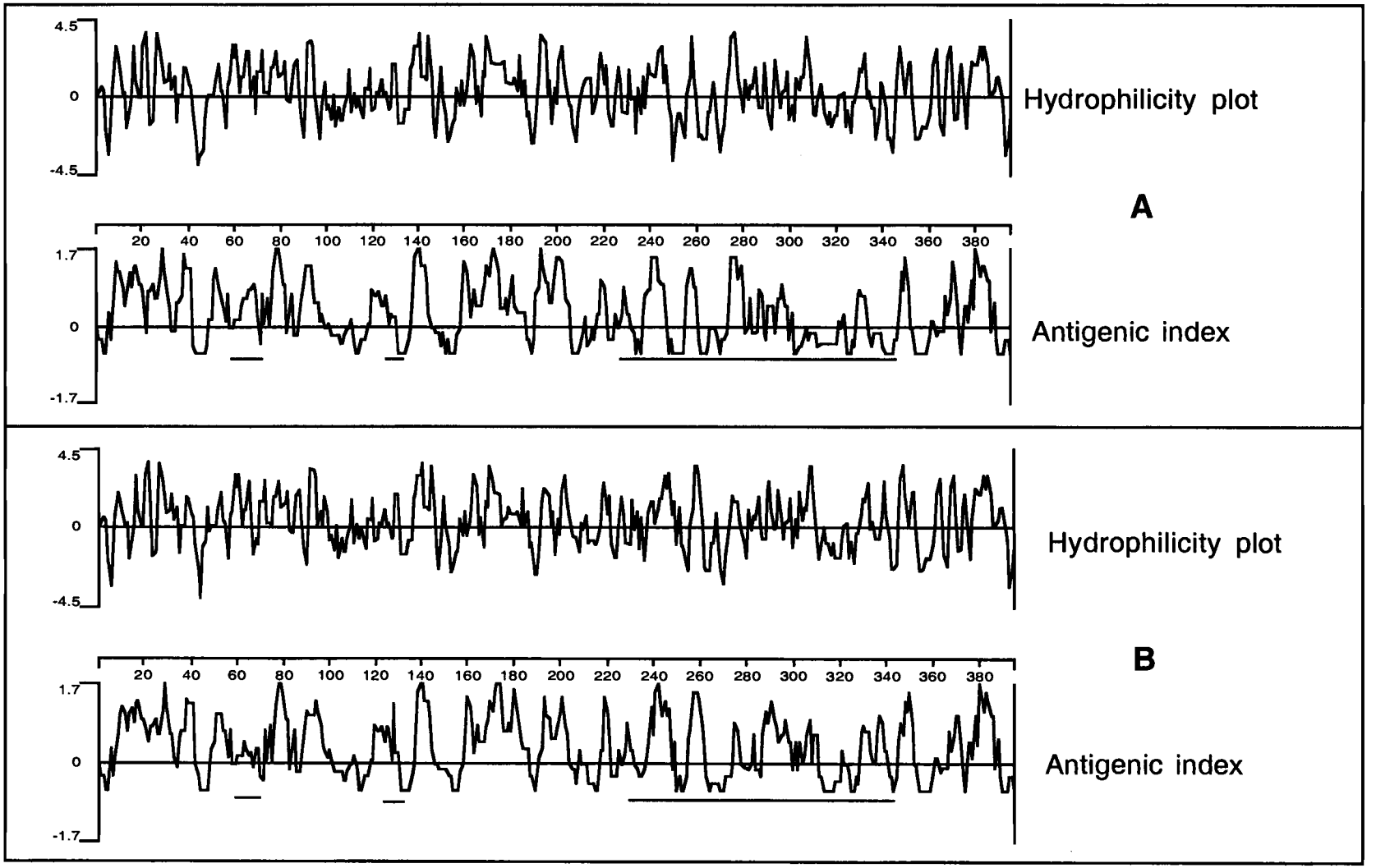
4.5
0
-4.5
Hydrophilicity plot
A
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
1.7
0
-1.7
Antigenic index
4.5
0
-4.5
Hydrophilicity plot
B
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
1.7
0
-1.7
Antigenic index

Figure 6.6

Table 6.3 Monoclonal reaction patterns of IPNV in an immunodot assay. Each abbreviated name in IPNV-isolate column designate the following: Jasper-parent = parent IPNV-Jasper; Jasper-RTS-S = progeny Jasper-RTS sensitive clones; Jasper-RTS-R = progeny Jasper-RTS resistant clones.

	Monoclonal Antibodies (Epitopes)										
IPNV-Isolate	AS-1	W-1	W-2	W-3	W-4	W-5	E-1	E-2	E-3	E-5	E-6
Jasper-ATCC	+	+	+	+	+	+	+			+	
Jasper-parent	+	+	+	+	+	+	+			+	
Jasper-RTS-S	+	+	+	+	+	+	+			+	
Jasper-RTS-R	+						+			+	+
VR-299	+			+	+	+	+			+	+
West Buxton	+	+	+	+	+	+	+			+	+
Ab	+	+	+				+	+		+	+
Canada-1	+						+	+		+	+
Canada-2	+									+	+
Epitope on	VP-2	VP-2	VP-2	VP-2	VP-2	VP-3	VP-3	VP-3	VP-2	VP-3	VP-3

DISCUSSION

Modern molecular techniques have revealed that populations of serologically identical RNA viruses are extremely heterogeneous (Steinhauer & Holland 1987). This is thought to be due to an inefficient nucleic acid repair system which retains transcription errors at a high rate compared to those in DNA. Despite of heterogeneity within virus populations, high mutation rates do not always imply rapid evolution,. Wild-type virus can predominate through an extensive passage history even though one variant can be dominant at any one time. Our lab has found that the epitope pattern of IPNV proteins VP2 and VP3 have been stable during multiple viral passages *in vivo* and *in vitro* over long periods of time. For example, virus isolated between 1970 and 1999 from chronically infected brook trout at a facility from which the A_1 archetype virus (ATCC VR-877) was originally obtained were identical in their epitope pattern with 11 monoclonal antibodies (Reno 1999). Likewise, virus isolated from rainbow trout from the mid-1960's until 1999 at a rearing facility in the Hagerman Valley, Idaho had an epitope pattern identical to ATCC-VR1430 (Buhl) (Reno 1999). However, these antigenic test have not been conducted for clones of the virus population. In this series of experiments, we found significant heterogeneity among clones with respect to epitope configuration and RTS sensitivity. As indicated in Figure 6.1 and Table 6.3, 92% of 50 clones were wild-type, RTS sensitive, and had the 11 epitopes charateristic of the Jasper serotype, all characteristic of the parental strain. However, 8% of the clones were a variant, Ja-R, and had an epitope configuration most closely related antigenically to the A_7, Canada 1 serotype.

Ja-S had the monoclonal reaction pattern typical of IPNV-Jasper strain, reacting with 5/5 epitopes found on West Buxton (A_1) and 2/5 epitopes found on Ab (A_3) serotype viruses (Caswell-Reno 1989). However, Ja-R had a different monoclonal reaction pattern which was similar to the monoclonal antibody reaction pattern of serotype Canada 1 (Table 6.3). Typical IPNV-Canada 1 serotype has positive monoclonal reactions to A_3 epitopes: E1, E2, E5, and E6, but no reaction to A_1 eptopes (W1-W5). Ja-R showed positive reactions at E1, E5, and E6 but not at E2. Even though Ja-R did not show exactly same epitope pattern as C1 (A_6), based on the data collection for serotypes of IPNV isolates that our lab have collected, the monoclonal reaction of Ja-R was identical to one isolate, IPNV-NEL, belonging to Canada 1 serotype. IPNV isolates belonging to Canada 1 have the ability to replicate in CHSE-214 cells but not in epithelioma papulosum cyprini (EPC) cell line whereas C-1 and Buhl do not (Fijan et al. 1983) (Ögüt 1995). No growth in EPC cells may be associated with the loss of epitope W4, since VR-299 has that epitope and grows in EPC cells. So we tested viral growth in both cell lines, CHSE-214 cells and EPC cells. Ja-S replicated in both cell lines; however, Ja-R replicated only in CHSE-214 but not in EPC cells. These results indicate that Ja-R has some of the same *in vitro* characteristics as Canada 1 serotype. The above results are interesting because the original IPNV-Jasper strain was isolated in Alberta, Canada and IPNV-NEL was isolated from the east coast of New Brunswick in Canada. Canada 1 serotype viruses have been found only on the east coast of Canada. Thus, these results are surprising in their diversion from the original Ja isolate and might have one of three explanations: first, exogenous contamination of the parent of our clones with a C1 serotype isolate (IPNV-NEL); second, the presence of heterogeneous serotypes of IPNV-Jasper in the preparation

which were replicating at similar rates; third, the selection of a viral variant under the selective pressure of the RTS inhibitor.

In terms of exogenous contamination, our lab has not used the IPNV-NEL isolate for the last five years including the time during which this experiment was carried out. In addition, the virus was passaged through brook trout twice prior to the cloning. Thus, it is highly unlikely that these clones contained laboratory contaminants.

It is difficult to determine if there were hetero-serotypes in the hatchery, Alberta, Canada where the original IPNV-Jasper was obtained. Two laboratories obtained IPNV, designated "Jasper", from the same hatchery, and Berthiaume et al (1992) reported Ja-D and Ja showed difference in monoclonal reaction and genetic information. They found different monoclonal antibody reactions in VP2 but not in VP3. The protype of IPNV-Ja (A_9 serotype of Hill & Way) was used in our experiments. However, the virus used by others was isolated from RBT, while the serotype was obtained from same hatchery, Aberta, Canada. Under the conditions of long term, chronic IPN infection which occur at some facilities, it may not be uncommon for multiple serotypes of IPNV to be present at a single facility. For example, from a facility located in northern Idaho, which is close to the Malgne River Hatchery, Alberta, four subtypes of IPNV were isolated between 1986 and 1996: Jasper (A_9), West Buxton (A_1), Buhl (A_1), Ab (A_3) (Reno 1995). In 1990 alone, viruses belonging to 3 serotypes were isolated at the same facility. Also at Wizard Falls Hatchery in Oregon two different subtypes were isolated from rainbow trout (VR-299) in 1975 and brook trout (Buhl) in 1974. Yamamoto (1975a, 1975b) also reported that both rainbow and brook trout were found to harbor IPNV at Malgne River Hatchery,

Alberta. This could support the possibility of the presence of heterologous IPNV in the hatchery in Alberta where the original isolations were made.

There is some precedent for a genetic shift under the influence of antibody. Wang (1992) reported monoclonal antibody neutralization-resistant variants from a population of IPNV-WB. Two variant strains were very different than parental West Buxton strain based on genomic data and serotype; neutralization-resistant strains had diverged from West Buxton, and more closely resembled members of the Sp (A_2) serotypes. Neutralization-resistant strains shared less than 80% sequence homology with West Buxton strains. Thus, it is possible that variant isolate become dominant under the influence of certain conditions.

We found parental Ja and Ja-S had the same monoclonal reaction pattern (Table 6.3). If the parental IPNV-Jasper contained a subpopulation of 8 % of Ja-R variants, E-6 monoclonal antibody reaction might be expected to be positive since Ja-R has the epitope, whereas Ja does not. So the question arose whether all Ja-R had same epitope pattern, including E-6. We tested all 4 Ja-R clones and found that all showed the same epitope pattern (data not shown). Originally the concentration of parent Ja tested for serotyping was $10^7 TCID_{50}$/ml, thus the concentration of Ja-R was approximately $10^6 TCID_{50}$/ml. We found that monoclonal reaction for E6 was not positive at a concentration of $10^6 TCID_{50}$/ml. Our laboratory experience has shown that Ja does not generally grow to a high titer (approximately 10^7 $TCID_{50}$/ml) (Ögüt 1995, Reno personal communication). However, in the past the titer used for MAb were above $10^7 TCID_{50}$/ml and no E6 showed up. For the serotyping of Ja-R, the clones were passaged once more in RTG-2 cells in the presence of RTS and the virus titer was significantly elevated (10^9 to

10^{10} $TCID_{50}$/ml) and produced a strong monoclonal reaction at E6. This information indicates that the replication of Ja-R might be excelled by that of Ja-S if the two strains replicate together. It is known that parental wild-type RNA phage consistently outgrew variant clones under normal *in vitro* conditions (Domingo et al 1978). Maret (1997) also found that one type of IPNV (Buhl) outgrew an other type of IPNV (West Buxton) in a superinfection experiment in brook trout. However, the stability between wild-type and variant virus could be changed by some condition such as interference by DI particles, different host or cell types, or immune selection (Steinhauer and Holland 1987) and thereby enhance the replication of a normally slower replicating virus. Especially, host immune selection can be a strong factor in driving virus evolution (Clements et al 1980, Palese et al. 1982, Webstrer et al. 1982). Even though we do not know the whole history of the Jasper-isolate that was used in this experiment, it has been passaged many times through trout as well as different cell lines in our lab. Additionally, we passaged the virus two times through brook trout just before this experiment, which could immunologically select more RTS-resistant strains from the virus population. Even though a 1:100 dilution of brook trout serum did not significantly inhibit the virus *in vitro* (Park unpublished result), this would be one of possible reasons how RTS-resistant population could emerge and comprise a relatively high proportion of the clones (8%) *in vitro*.

We found Ja-R had an epitope composition most closely related to the Canada 1 serotype. However, there is some question as to how similar Jasper-RTS resistant clones are to Canada 1 at the gemomic level. No published information is available about the sequence of the VP2 region of IPNV-Canada 1. Even though Heppell et al. (1993) reported the sequence of a 310bp cDNA fragment of IPNV-Canada 1, this fragment was

at the junction between pVP2 and NS coding regions, and the region does not overlap with the VP2 region we sequenced. However, they found Canada 1 and Canada 3 showed 100% homology and Canada 1 had high percentage of amino acid homology with IPNV-Ab (95.12%) in this region and only 84.5% homology with C2. We also found that Ja-R had highest amino acid homology (88.1%) with Ab and 85.7% homology with Canada 2 (Table 6.2). Thus, we might assume that Ja-R might have high homology at the gemomic level with IPNV-C1 but this would require the sequencing of the C1 genome.

However, discrepancy between serogroup and genogroup has been reported in IPNV previously (Heppell et al. 1993). The earliest recognized, VR-299, was 100% homologous with Ja-D (Duncan & Dobos 1986, Heppell et al. 1993) and 99.7% homologous with Ja (Ma 1996). However the serotypes of these isolates are different (Caswell-Reno 1989; Hill and Way 1995). Using reciprocal cross neutralization, Hill and Way (1995) determined that Jasper (A_9) was most closely related to Sp (r=25), West Buxton (r=37), and Ab (r=56) but was markedly different from C1 (r=854). Using 11 monoclonal antibodies in an immunodot test, Jasper differed from West Buxton by a single epitope (E6), from Sp by 4 epitopes (W1, W2, W4, W5), from Ab by 5 epitopes (W3, W4, W5, E2, E6), and from C1 by 7 epitopes (W1, W2, W3, W4, W5, E2, E6) (Caswell-Reno 1989). The results in this paper also, in part, support the differences. Based on their monoclonal reactions, serotype of C1, C2, and C3 are more closely related to each other than to serotype Ab. IPNV-Ab has two more monoclonal reactions against VP2 than C1, C2, and IPNV-C3. Ja-R has exactly the same monoclonal reaction as Canada 2 based on VP2 reactions: 0/5 epitopes known to be on VP2 were positive. However the amino acid sequence of Ja-R showed a closer homology with IPNV-Ab

(88.1%) (Heppell et al. 1993) than IPNV-Canada 2 (85.0%) (Heppell et al. 1993). Few sequences are available for the whole segment A encoding both VP2 and VP3, responsible for serotype. Thus, we are limited in comparing the relationship between serotype data and genotype data. However the differences between serotype and genomic information indicates that the fact that Ja-R is similar to C1 serotype does not always indicate contamination with serotype C1.

Glycosylation as well as proper folding of the protein is another important factor in antigenicity (Caust et al. 1987). Even though contradictory results have been noted (Perez et al. 1996, Nicholson personal communication), the possibility of glycosylation in IPNV has been suggested (Estay et al. 1990, Hávarstein et al. 1990, Hjalmarsson et al. 1999,Espinoza et al. 2000). To date, two possible glycosylation sites, N-glycosylation (Estay et al. 1990) and O-glycosylation (Hjalmarsson et al. 1999), have been suggested. In this study, possible glycosylation sites for N-glycosylation and O-glycosylation were deduced from amino acid sequences of two IPNV-Jasper strains. The two strains had same number of possible N-glycosylation sites, Asn(N)-X-Ser(S)/Thr(T) (Figure 6.3). Three sites were present on the same regions of two strains (63-65,108-110, 224-225), however the possible sites at 63-65 had different hydrophilic characteristic: Ja-S was hydrophilic at this region, however Ja-R was not. The last possible site was present at different regions: Ja-S amino acids 242-244 was a hydrophilic region, while Ja-R amino acids 233-235 were not hydrophilic. The two strains had similar numbers of possible O-glycosylation sites involving the amino acids, Ser or Thr. RTS sensitive strain had 69 possible sites, while the Ja-R had 72 sites among 394 amino acid residues. Ja-S had 14 possible O-glycosylation sites on hydrophilic residues, while, Ja-R had 10 sites. This

indicates that the Ja-S strain has more possible glycosylation sites for both N-glycosylation and O-glycosylation on hydrophilic regions than Ja-R does. This is interesting because our biochemical studies indicated that the RTS inhibitor against IPNV could be a lectin (Park unpublished data). If lectins inhibit IPNV, glycosylation and glycosylation sites would be important factors in viral agglutination. However, we need more data to determine viral glycosylation and the possibility of agglutination by lectins against IPNV.

In conclusion, clones from IPNV-Jasper which are sensitive to RTS were heterogeneous with respect to the degree of sensitivity. The clones with altered sensitivity also had altered genome sequences and epitope conformation. Further work needs to be done to determine the mechanisms and causes of these alterations.

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CHAPTER 7

THESIS SUMMARY

In this thesis, three major aspects of the interaction between normal RTS and IPNV were studied: the characteristics of an inhibitor of infectious pancreatic necrosis virus (IPNV) found in normal rainbow trout serum; the effect of *in vitro* viral passage on virulence and sensitivity of IPNV to RTS; and genetic differences between RTS sensitive clones and RTS resistant clones of IPNV.

The studies dealing with the characteristics of the RTS inhibitor and its mechanism of inhibition allowed us to narrow the possible nature of inhibitory molecules to a "6S" inhibitor in RTS. The strongest virus inhibition was obtained at a size of approximately 150 kDa. The inhibitory activity was greater in the presence of cations, either Ca^{2+} or Mg^{2+}, than in the absence when it was partially purified. It was stable at temperatures up to 50°C and at pH between 4–10. The inhibition was neither related to prior cell induction nor involved in masking of a viral receptor. The inhibitor acted directly on the virus, which was more strongly inhibited when RTS was present during viral replication than when the virus was simply pretreated. A series of experiments indicated that phenotypic characteristics of the virus may be important factors in its sensitivity to inhibition by RTS. Sensitivity of virus to RTS was altered by the cell line in which virus was produced. The inhibition level was dependent on the length of time during which virus was exposed to RTS, with inhibition maintained for at least 16h postinfection. The apparent inhibition level of RTS was dependent on cell density: at a

cell density $\leq 2x10^5$/ml, inhibition was insignificant ($10^{-1.1}$TCID$_{50}$/ml reduction); however, when cells were greater than $3x10^5$/ml, the apparent inhibition level was very high ($\geq 10^{-6.3}$TCID50/ml reduction). Virus inhibition was closely related to serum source and host cell specificity: IPNV was more efficiently inhibited by RTS in salmonid cell lines than in non-salmonid cell lines. Most of the salmonid sera tested were inhibiory, while non-salmonid sera did not inhibit IPNV replication on RTG-2 cells. Rainbow trout continued to show a significant level of inhibition starting 23 weeks post hatch

Some of the experiments mentioned above have shown that RTS sensitivity of virus was changed when it was replicated in different *in vitro* conditions. Thus, there was a question as to whether *in vitro* passage of IPNV with RTS effects on sensitivity to rainbow trout serum and on virulence. Three closely related isolates of IPNV (A_1 serotype, Buhl subtype) were passaged five times in RTG-2 cells with either MEM-10 or MEM-10 with 1% rainbow trout serum (MEM-RTS) and were tested for sensitivity to RTS *in vitro* and virulence *in vivo*. The RTS sensitivity of IPNV was changed by multiple viral passages, and this was dependent on IPNV isolate. It was found that IPNV isolate crayfish passaged in MEM-10 showed a markedly increased RTS sensitivity, up to $10^{-8.0}$ TCID$_{50}$/ml reduction in virus titer. However, we found highly increased RTS sensitivity was not correlated with decreased virulence. We found over all that no relationship was obtained between RTS sensitivity and virulence. Mortality level in brook trout fry was highly variable during viral passages, ranging between 30-89%. Sustenance of virulence during viral passage was dependent on IPNV isolate and culture conditions. All three isolates showed identical antigenicity patterns with a panel of 11 monoclonal antibodies, irrespective of viral passage conditions.

Even though IPNV-Jasper(Ja) used in most of the *in vitro* experiments was highly sensitive to RTS, there was a high variation in sensitivity to inhibition by RTS. Genetic differences were also found in three isolates. Thus, the question arose whether the infectious pancreatic necrosis virus isolate IPNV-Jasper (Ja) is homogeneous or heterogeneous with respect to inactivation by normal rainbow trout serum (RTS). Consequently, 50 clones were tested for RTS sensitivity. The RTS sensitivity of each clone ranged from completely resistant to highly sensitive (0-10^8 $TCID_{50}$/ml reduction). Eight percent of clones (4/50) were very sensitive to RTS (Ja-S) and eighty four percent of clones (42/50) showed a mid-range of RTS sensitivity. The final eight percent of clones (4/50) were RTS resistant (Ja-R). Enzyme immunodot assay revealed that Ja-S clones showed a monoclonal reaction typical of the parents, Ja; however, Ja-R clones differed by several epitopes from the parental strain. Analysis of the two strains revealed that Ja-S and Ja-R had significant differences in their nucleic acid sequences for the capsid protein VP2. These two strains shared 81% and 86% identity in nucleic acid and in amino acid sequences, respectively.

In conclusion, exposure of IPNV to RTS influenced replication and led to altered genetic and antigenic composition, but did not appear to be significantly correlated with virulence. The precise nature of the inhibitor molecule(s) remain(s) unknown, but it shares characteristics with lectins. Thus, future studies should address the mechanism and cause of these alterations, and the "evolution" of RTS sensitivity under *in vitro* and *in vivo* pressures from serum components. Finally, the determination of the potential lectins as RTS inhibitors of IPNV and the relationship between viral glycosylation and sensitivity of virus to RTS.

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