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Title: <u>PERSISTENT INFECTION OF SALMONID CELL LINES WITH INFECTIOUS</u>

<u>PANCREATIC NECROSIS VIRUS: A MODEL FOR THE CARRIER STATE IN TROUT</u>

Abstract approved: _____
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Persistent infections with infectious pancreatic necrosis virus
(IPNV) were established in two salmonid cell lines and in live brook
trout. Virus inocula containing high concentrations of defective
interfering IPNV enhanced but were not a requirement for the establish-
ment of persistent infections in cell lines derived from steelhead
trout (STE-137) and chinook salmon (CHSE-214) embryos. Persistent
infections were initiated in live brook trout by feeding a ration
containing IPNV.

Viral persistence was demonstrated by the detection of infectious
virus in cell culture fluids and carrier trout feces. A fluctuating
release of low levels of virus was characteristic of viral persistence
both <u>in vitro</u> and <u>in vivo</u>. Infectious virus was produced by 0.06 to
1.0% of the cells in carrier brook trout tissues and cell lines
persistently infected with IPNV. Viral persistence in these cell lines

was further characterized by the presence of viral antigens in 1% or less of the cell population. These cells were often in advanced stages of infection indicating that death results from viral replication. The remainder of the cells in the population is protected from superinfection with homologous but not heterologous viruses. Protection is disrupted by passages of these cells in growth medium containing anti-IPNV antibody. Extended propagations with high levels of antibody cures persistent infection.

Low levels of infectious virus released during IPNV persistence results from limiting the number of cells as well as the amount of infectious virus produced by persistently infected cells. Interferon, temperature sensitive and defective interfering viruses are known to control infectious virus production in certain persistently infected cell lines. Interferon and temperature sensitive virus were not detected during IPNV persistence in STE-137 and CHSE-214 cell lines in this study. Interferon was also not detected in carrier brook trout sera tested on rainbow trout (RTG-2) cells but this did not preclude its presence. The amount of infectious virus produced during viral persistence seems instead to be controlled by the production of defective interfering virus. Defective interfering virus was isolated from STE-137 cell culture persistently infected with IPNV by isopycnic centrifugation in CsCl. This virus had a density of 1.29 g/cc. Infectious virus (1.33 g/cc) was also produced by these cells. Defective interfering virus effectively suppressed the yields of infectious virus from control STE-137 cells infected with both viruses.

The production of defective virus was also supported by electron microscopic examinations of persistently infected STE-137 cells. These results indicated that the production of defective interfering IPNV by persistently infected cell lines may control infectious virus replication and prevent the cytocidal effect that usually is accompanied by IPNV infection. The similarities between viral persistence in these cell lines and carrier brook trout suggest that the same virus-host cell interactions may be involved both in vitro and in vivo. These cell lines can therefore serve as invaluable models for understanding persistent IPNV infections.

Persistent Infection of Salmonid Cell Lines with Infectious
Pancreatic Necrosis Virus: A Model for the Carrier State in Trout

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INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is a highly destructive pathogen of hatchery reared trout. Trout younger than 6 months are the most susceptible and experience high mortalities (Frantsi and Savan, 1971a). Unfortunately, the problem for the fish culturist does not end with the decline in virus-induced mortalities. Fish that survive the initial infection, especially those experiencing external signs, may become lifetime carriers shedding infectious virus in the fecal and sex products (Billi and Wolf, 1969). These carrier trout act as continual sources of infectious virus that can be transmitted vertically or horizontally to other fish in the population. The characteristics of the carrier state in these trout have been described by several investigators (Frantsi and Savan, 1971b; Reno et al., 1978 and Yamamoto, 1975a, 1975b) but the mechanisms underlying the maintenance of viral persistence are unknown.

The persistent nature of IPNV infections in cell cultures has only recently been reported (Ahne, 1977 and Hedrick et al., 1978a). These cultures were found to share certain characteristics with those described for carrier trout and suggested that they might serve as _in vitro_ models for viral persistence _in vivo_. The purpose of this study was to: (1) characterize the persistence of IPNV in these cell lines, (2) to determine the mechanisms that control infectious virus

replication and maintain viral persistence and (3) to examine certain

characteristics of carrier brook trout to determine if the same

mechanisms that maintain viral persistence _in vitro_ might also

function _in vivo_.

LITERATURE REVIEW

Historical Perspective

M'Gonigle (1941) was the first to describe a severe disease among alevin trout in which the mortalities exhibited an acute catarrhal enteritis. Histopathological examinations of brook trout (Salvelinus fontinalis) with identical signs revealed massive pancreatic necrosis (Wood et al., 1955). Snieszko et al. (1959) determined the infectious nature of the disease by reproducing the typical signs and mortality among brook trout fed ground viscera from infected fish. With the advent of salmonid tissue culture, Wolf et al. (1960) isolated and demonstrated the viral nature of the etiological agent.

Distribution and Host Susceptibility

The world-wide traffic of salmonid fish and eggs has contributed to the spread of infectious pancreatic necrosis virus (IPNV). Outbreaks of IPNV have been reported in the western U.S.A., Canada, Scotland, Sweden, Denmark, France and Japan.

Although IPNV may infect and persist in fish of several species (Hill, 1977), mortalities of epizootic proportion are restricted to brook and rainbow trout (Salmo gairdneri). Excellent reviews covering the distributions and host range of IPNV have been written by Scherrer (1973), Wolf (1976) and recently by Pilcher and Fryer (1980).

External and Internal Signs

The first indication of IPNV infection is a sudden increase in mortality accompanied by a violent corkscrew motion while swimming. Other typical external signs are darkening in color, abdominal distension, exopthalmia and hemorrhages at the base of the ventral fins (Wolf, 1966). Internally, the liver and spleen are pale and food is absent from the digestive tract. The stomach and intestine will contain a clear to milky mucous exudate. Petechiae of the pyloric caeca, a typical feature of outbreaks among fish reported in N. America, is not always observed in European trout (McKnight and Roberts, 1976).

The target organ of IPNV appears to be the pancreas (Wolf and Quimby, 1971) where extensive damage to the acinar cells is accompanied by an acute enteritis. Cells sloughing from the intestinal epithelium combining with excess mucous contribute to the whitish exudate characteristic of the disease. Lesions in the pancreas of fish surviving initial IPNV infections become less obvious with increasing age and areas of acinar degeneration are replaced by fibroblastic tissues (McKnight and Roberts, 1976). Although externally asymptomatic, degenerative changes in the pancreas, spleen and intestine continue but decrease in severity as the fish increases in age. Virus can be isolated from trout with these clinical signs and the persistence of IPNV replication in these tissues contributes to the maintenance and duration of the carrier state.

The Carrier State

Among the fish surviving IPNV epizootics, some may become lifetime carriers, shedding infectious virus in fecal and sex products to the surrounding environment (Billi and Wolf, 1969). Yamamoto (1975a) has reported carrier rates approaching 90% among brook trout from hatcheries where IPNV was endemic. The age at which brook or rainbow trout are exposed to IPNV determines the duration of the carrier state. Reno et al. (1978) found that alevin rainbow and brook trout populations which experienced symptoms and mortalities were likely to become carriers for at least one year. In contrast, adult brook and rainbow trout injected with IPNV developed an immune response and were only transiently infected. Virus shedding decreased with the appearance of increasing anti-viral neutralizing antibody levels until IPNV was no longer detectable in the feces, kidney or gonads of these fish (Wolf and Quimby, 1969). The difference in the response of adult and alevin trout to IPNV infection may involve immune tolerance or immunocompetence host mechanisms. Similar viral persistence has been observed between adult and infant humans infected with cytomegalovirus, measles and progressive rubella viruses (NIAID task force report, 1979). Trout which can develop an effective immune response become only transiently infected while young trout unable to respond become persistently infected. The kidney, spleen and caeca are the organs of choice for the recovery of IPNV from carrier trout which can be sacrificed (Yamamoto and Kilistof, 1979 and Reno et al., 1978). Lower titers

of virus are released in the feces and sex products of carrier trout. Their release can contribute to the horizontal and vertical transmission of IPNV.

Physical, Biochemical and Serological Characteristics

Physical

The naked icosahedral capsid of IPNV is composed entirely of protein. This protein coat determines the antigenic character as well as the resistance of IPNV to physical and chemical inactivation. The stability of IPNV to changing environmental conditions may contribute to its persistence in hatchery and feral trout populations. Desaultes and Mackelvie (1975), demonstrated that IPNV could retain 0.001% of its original infectivity when stored at pH 2 or pH 9 for 5 weeks. Infectious pancreatic necrosis virus was stable to drying at room temperature for a period of 5 weeks after which time infectivity declined rapidly. Chemical agents such as ozone, chlorine and iodine are suitable for the inactivation of IPNV when adequate levels can contact the virus. A report by Bullock et al. (1976) of infectious pancreatic necrosis (IPN) among brook trout hatched from eggs treated with 100 ppm Wescodyne[R] (1.6% available iodine) demonstrated that surface decontamination may be difficult.

Lightner and Post (1969), reported a diameter of 57 nanometers (nm) for virions found in thin sections made from infected trout tissues. Negatively stained virus purified from rainbow trout (RTG-2) cultured cells had a mean diameter of 74 nm (Kelley and Loh, 1972).

The icosahedral capsid is composed of 180 structural subunits arranged to form 92 capsomeres (Cohen and Scherrer, 1972) surrounding a viral genome known to contain two unique double-stranded segments of RNA.

Biochemical

The ability to purify IPNV by isopycnic banding in cesium chloride (CsCl) gradients (density 1.33 g/cc) has allowed the biochemical analyses of the virions nucleic acids and proteins. Dobos (1977) and MacDonald and Yamamoto (1976) have demonstrated that the two segments of RNA are double-stranded and have molecular weights of 2.3 and 2.5 X 10^6 daltons. In addition, these segments have been shown to be unique species by oligonucleotide fingerprinting (MacDonald et al., 1977). The RNA of IPNV comprises 8.7% of the virions mass, the remainder is protein (Dobos et al., 1977). The viral proteins are distributed into three size classes of polypeptides as determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Dobos, 1976). Cohen and Scherrer (1974) reported seven distinct polypeptides but more recent investigations by Dobos (1977) and Chang et al. (1978) agree on the presence of four major polypeptides in purified virions. The polypeptides with molecular weights of 55,000, 30,000 and 27,000 daltons are the major components of the capsid and the 90,000 dalton internal protein is the virion associated transcriptase. The external capsid proteins are responsible for the different antigenic characteristics of IPNV.

Antigenic Diversity

Lientz and Springer (1973) compared 42 isolates of IPNV by neutralization with a polyvalent antiserum directed against three North American and two European strains. They concluded that at least seven distinct strains of IPNV were present among the isolates tested. Other studies indicating serological diversity among IPNVs isolated from salmonids have been reported (Wolf and Quimby, 1971; Vestergard, Jorgensen and Kehlet, 1971 and Malsberger and Cerini, 1965).

Several IPN-like viruses, some of which show a serological relationship to IPNV, have been isolated from a wide range of nonsalmonid hosts. These viruses, as well as IPNV, were once classified as reo-like but increasing biochemical and physical evidence suggest that they all should be placed in a new taxonomic group of RNA viruses. Although not formally accepted, Chang et al. (1978) have proposed the name diplornavirus for this group. Unlike reoviruses, the IPN-like viruses contain only two segments of double-stranded RNA instead of ten, a single capsid instead of an inner and outer capsid (Dobos et al., 1977) and have a lower density in CsCl. Infectious pancreatic necrosis-like viruses have been isolated from bivalve molluscs (Tellina tenius) (Underwood et al., 1977), infectious bursal disease (IBDV) in chickens (Gallus gallus) (Muller et al., 1979), drosophila (Drosophila melangoster) (Teninges et al., 1979) and European (Anguilla anguilla) and Japanese (Anguilla japonica) eels by Sano (1976). The viruses of the bivalve mollusc and IBD of chickens have been partially characterized biochemically and have similar nucleic acid, protein, particle diameter, bouyant density and morphology

to IPNV. The viruses from drosophila (Drosophila X virus) and Eel

Virus European (EVE) from eels have not been characterized but based

on particle morphology and serological relationships are similar to

IPNV.

Formation of Defective Interfering Virus

A number of cell lines of salmonid and nonsalmonid origin replicate

IPNV to titers approaching 10^9 plaque forming units (PFU) or $TCID_{50}$

per ml of culture fluid. However, Malsberger and Cerini (1963) and

later Nicholson and Dunn (1974) found that repeated passages of IPNV at

high multiplicities of infection substantially decreased the yields of

infectious virus. The reduction in yield was attributed to the formation

of defective virus which resulted in homologous interference. The ability

of IPNV to produce defective interfering (DI) virus in vitro was also

confirmed by MacDonald and Yamamoto (1978) who found that interfering

virus co-purified with infectious virus.

Evidence for the in vivo production of DI viruses was reported by

Nicholson and Dexter (1975). Homogenized tissues from carrier brook

trout inoculated onto RTG-2 cells at low dilutions resulted in no

cytopathic effect (CPE), while at higher dilutions typical CPE and virus

yields were obtained. These DI viruses were characterized by their

ability to interfere with the replication of infectious virus. This

interference may prevent the cytocidal effect of infectious virus

production. Interferon which might have prevented infectious viral

replication in these cells was not detected (MacDonald and Yamamoto,

1978) and suggested that DI viruses were responsible for cell survival.

A variability in the production of DI viruses by certain IPNV strains has been examined by MacDonald (1978). Strains producing DI virus have been correlated with a unique 'ringed plaque' morphology while nonproducing strains form a clear plaque. 'Ringed plaques' result from the survival of cells at the perimeter of the enlarging plaque. These cells may survive as a result of the interference of DI with the replication of infectious virus.

Persistent Viral Infections in Cell Culture

Persistently infected or carrier cell cultures have been established with members of most of the major taxonomic groups of RNA viruses (Rima and Martin, 1976). Persistently infected cell cultures are characterized by the continued growth and division of cells in the presence of a virus which may normally be highly cytocidal. These cultures have contributed to understanding the interactions between host cell and virus that result in persistent infection. The mechanisms by which viruses persist in cell cultures have also been found to operate in the intact animal (Huang and Baltimore, 1970). These cultures can therefore serve as important _in vitro_ models of viral pathogenesis _in vivo_ and provide better understanding of the carrier state.

The ability of viruses which are normally cytocidal, to maintain persistence in cell culture is not completely understood. At least three mechanisms for viral persistence have been proposed: (1) involvement of defective interfering virus, (2) selection of mutant viruses and/or (3) integration of the virus genome into the host's chromosome (Rima and Martin, 1976).

Huang and Baltimore (1970) were the first investigators to describe the properties of DI virus and their possible role in persistent infection. These viruses contain the normal complement of viral proteins but lack an essential part of the genome which renders them noninfectious. In addition, DI viruses have the ability to interfere with the replication of infectious virus by unexplained mechanism(s) (Huang, 1977). The production of both DI and infectious virus by persistently infected cell cultures has been proposed as the method by which the cytocidal course of viral replication is arrested and persistence maintained (Palma and Huang, 1974; Holland and Villareal, 1975a). Joklik (1977), has characterized persistence involving DI particles as follows: (1) cells are rather nonpermissive and virus yields are small; (2) only a small fraction of the population is infected; infected cells do not divide but die; (3) interferon or interfering virus are present in the medium; (4) antiviral antibody in large amounts cures persistent infection; and (5) clones of uninfected cells are easily obtained by cloning cells in an antibody-containing medium.

Mutant viruses especially those which are temperature sensitive (ts) are known to be important in the initiation as well as the maintenance of persistent infection. Younger et al. (1976) found that certain ts mutants of VSV could initiate persistence in L cells without the help of homologous DI virus which were required when wild type (WT) VSV was used. In addition persistent infections initiated with WT VSV

and DI viruses were characterized by the increased production of ts

mutants which eventually became the sole virus type released.

Similar results have been obtained with persistent infections of reo-

virus in L cells (Ahmed and Graham, 1977). Temperature sensitive

mutants have the ability to interfere with infectious virus replication

and therefore behave as conditional DI virus (Younger and Quigliana,

1976). The overall effect was to arrest cell lysis and allow viral

persistence.

The integration of viral genomes into the host cells chromosome

has been suggested as a possible mechanism for the persistence of

certain RNA viruses which do not exhibit reverse transcriptase activity.

Simpson and Iinuma (1975) and Zhadnov (1975) have reported the recovery

or detection of proviral DNA from mammalian cells persistently infected

with respiratory syncitial virus, measles, sindbis and tickborne

encephalitis virus. Additional studies are required before it will

be possible to evaluate the role of these proviral integrations on

persistent infections in vitro or in vivo. Excellent reviews concerning

viral persistence in cell cultures have been written by Martin and

Rima (1976) and Joklik (1977).

Persistent Infections of Salmonid Cell Lines with IPNV

Persistent infection of salmonid cell lines were reported by both

Ahne (1977) and Hedrick et al. (1978a) working independently. In both

studies persistence was demonstrated by (1) the release of infectious

virus (2) detection of viral antigens by immunofluorescence, or double-

stranded RNA by acridine orange staining and (3) resistance to super-
infection. Hedrick et al. (1978b) further characterized the persistence
of IPNV in steelhead trout (STE-137) cells and suggested that the
concurrent production of DI and infectious virus might be responsible
for maintenance of persistent infection. MacDonald et al. (1979) has
also examined the persistence of IPNV in CHSE-214 cells and found
similar results to those reported previously by Ahne (1977) and
Hedrick et al. (1978b).

MATERIALS AND METHODS

Cell Lines

Steelhead trout (Salmo gairdneri) STE-137 and chinook salmon
(Oncorhynchus tshawytscha) CHSE-214, two continuous cell lines derived
from embryos (Fryer et al., 1965) were used in this study (Table 1).
Both cell lines support the replication of several fish viruses and are
routinely used for diagnostic purposes. The CHSE-214 cell line was
established in 1964 and has undergone 350 transfers since its origin.
The STE-137 cell line has been transfered 250 times since their
establishment in 1963. Both cell lines can be stored for long periods
(at least 14 years) in liquid nitrogen and demonstrate the same
epithelial morphology upon subsequent culturing. The RTG-2 cell line
used in this study was derived from gonadal tissues of rainbow trout by
Wolf and Quimby (1962). This cell line was used between transfers 120-
150 and demonstrated a characteristic fibroblastic morphology.

Cell lines were propagated in Eagle's minimum essential medium
(MEM) with Earle's salts (Autopow, Flow Laboratories Inc.). The
medium was supplemented with either 5% (MEM-5) or 10% (MEM-10) fetal
bovine serum (Flow Laboratories Inc.), penicillin 100 I.U./ml and
streptomycin 100 μg/ml (Grand Island Biological Co.). Cells were grown
at 16°C in plastic culture flasks (Corning Glass Works) or 32 oz.
prescription bottles (West Co.).

Table 1. Cell lines used in this study for the propagation of infectious pancreatic necrosis virus.

Cell Line	Host Tissue Derived From	No. of Transfers	Morphology	Virus Susceptibility	Reference
CHSE-214	Chinook salmon embryos	250-300	epithelial	IPNV[b], INHV HS, CSV[c]	Fryer et al., 1965
STE-137	Steelhead trout embryos	150-225	epithelial	IPNV, IHNV HS[c], CSV[c]	Fryer et al., 1965
RTG-2	· Rainbow trout gonad	120-150	fibroblastic	IPNV, IHNV HS	Wolf and Quimby, 1962

[a]Wolf, K. and J. A. Mann, 1980.

[b]Abbreviations as follows: Infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), Herpesvirus salmonis (HS) and chum salmon virus (CSV).

[c]Susceptibility to these viruses were determined in this study.

Viruses

Infectious Pancreatic Necrosis Virus (IPNV)

Five IPNV and one IPN-like viruses were used in this study (Table 2). The Cascade Locks (CL-IPNV) and cutthroat trout (CTT-IPNV) strains were isolated from asymptomatic coho salmon (Oncorhynchus kisutch) and cutthroat trout (Salmo clarki), respectively (McMichael, 1974 and McMichael et al., 1975). An isolate from brook trout designated VR-299 was used as a reference strain (Wolf, 1966). The Buhl and French-21 strains of IPNV were isolated from rainbow trout and have been described by Lientz and Springer, (1973). Eel Virus European (EVE) was isolated from moribund eels reared on Japanese eel farms by Sano (1976).

Infectious Hematopoietic Necrosis Virus (IHNV)

Metolius River strain of IHNV isolated from spawning adult kokanee salmon (Oncorhynchus nerka) was used as a reference. This virus was isolated in 1978 and determined to be IHNV following neutralization with known anti-IHNV antisera. In addition, two other fish viruses were used. Herpesvirus salmonis was isolated from rainbow brood stock and characterized by Wolf et al. (1978). Chum salmon virus (CSV) was isolated from asymptomatic sexually mature adult chum salmon (Oncorhynchus keta) from the island of Hokkaido, Japan by J. R. Winton, 1978.

Table 2. Viruses used in this study to establish and examine persistent infections with infectious pancreatic necrosis virus.

Virus (strain)	Host Isolated From	Location	Reference
CL-IPNV	Coho salmon	Cascade Locks, OR U.S.A.	McMichael, 1974
CTT-IPNV	Cutthroat trout	N. Santiam River, OR U.S.A.	McMichael et al., 1975
VR-299 IPNV	Brook trout	Leetown, WV U.S.A.	Wolf, 1966
Buhl IPNV	Rainbow trout	Buhl, ID U.S.A.	Lientz and Springer, 1973
French-21 IPNV	Rainbow trout	d'Honnincthum, France	Lientz and Springer, 1973
EVE	European eel	Japan	Sano, 1976
Metolius River IHNV	Kokanee salmon	Metolius River, OR U.S.A.	Mulcahy, 1977
Herpesvirus salmonis	Rainbow trout	Winthrop, WA U.S.A.	Wolf et al., 1978
CSV	Chum salmon	Hokkaido, Japan	Winton, J. R. (personal communication)

Preparation of Virus Stocks

Both IPNV and IHNV stock viruses were prepared by inoculating monolayers of CHSE-214 cells in 150 cm^2 flasks. Viruses seeded at low multiplicities of infection (MOI=.01) were adsorbed to monolayers for 1 h at 16°C. Following adsorption, 30 ml of MEM-5 was added and the flask sealed. The medium containing virus was harvested when it was determined that CPE was extensive, usually 5 days for IPNV and 8 days for IHNV. Cellular debris was removed by centrifugation at 2000 x g for 10 min. Virus was dispensed in 1.0 ml aliquots and frozen at -60°C. The titer of IPNV stocks ranged from 0.1-1.0 x 10^9 PFU/ml. Stocks of the Metolius River strain of IHNV contained 2.0 x 10^7 PFU/ml.

Assay of Infectious Virus

Tissue Culture Infective Dose (TCID$_{50}$) Assay

The tissue culture infective dose (TCID$_{50}$) assay was used to quantify virus concentrations in feces, tissues and supernatants. Serial ten-fold dilutions of the suspension containing virus were made in MEM-0. Each virus dilution was transfered to a multiwell tissue culture dish (Flow Laboratories, Inc.) so that each of 8 wells received a 0.1 ml aliquot. A suspension of the desired cell line was prepared to a density of 10^6 per ml in MEM-10 and one drop of this suspension was added to each well of the dish. Dishes were sealed with a mylar film and the cultures incubated at 16°C for 5 to 10 days. The number of wells demonstrating CPE were recorded and the 50% endpoint calculated by the method of Reed and Muench (1938).

Monolayer Plaque Assay

The monolayer plaque assay was used for enumerating concentrations of virus in suspensions and plaque purification of virus preparations (Wolf and Quimby, 1973). Either 25 cm^2 culture flasks (Corning Glass Works) or 24 well culture dishes (Falcon Plastics) were seeded with cell suspensions and incubated until the monolayer approached 90-95% confluency. Serial tenfold dilutions of virus were prepared in MEM-0. The growth medium surrounding the cells was removed and 0.2 ml or 0.1 ml aliquots of each viral dilution were added to replicate monolayers. After a 1 h adsorption period on a rocking platform (Belco Co.), a nutrient overlay was added.

Agarose overlays were prepared by mixing equal volumes of double strength (2X) MEM-5 and 1.6% agarose (Seakem Inc.). Methylcellulose overlays were prepared in a similar manner but instead of agarose, 2X MEM-5 was added to an equal volume of 2% methylcellulose (Fisher Scientific Co.). The assay was terminated after 3 days for IPNV and 8 days for IHNV. For plaque purification, neutral red (1:1000) was added 12 h prior to picking plaques. Plaques were counted after fixing cell monolayers in formaldehyde (37%) for 1 h and staining with 1% crystal violet for 5 min. Replicate flasks or wells containing 20-200 plaques were counted, averaged and recorded as PFU/ml.

Preparation of Rabbit Anti-Virus Serum

Plaque Purification of Virus Isolates

Three times plaque purified CL-IPNV and CTT-IPNV were used to raise antisera in rabbits. Well-defined plaques were selected, an agar plug removed from the center and resuspended in MEM-0. Tenfold dilutions of the virus suspension were placed onto new cell monolayers and the plaquing procedure repeated until a total of three purifications were obtained.

Preparation of Virus For Injection

Plaque-purified virus were grown in mass culture in CHSE-214 cells and separated from cellular components by two CsCl gradients. After the initial 18 h centrifugation (114,000 x g) the band containing virus was withdrawn by side puncture of the nitrocellulose centrifuge tube. The band containing virus was dialyzed against TNE (0.01 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.001 M EDTA) buffer for 1 h at 4°C and layered over a second CsCl gradient and centrifuged to equilibrium. The band containing virus was withdrawn, dialyzed against TNE and examined for total infective virus by $TCID_{50}$ analysis. The titers of virus contained in the bands routinely ranged from $10^{10.5}$ to $10^{11.3}$ $TCID_{50}$ per ml.

Injections of Rabbits and Collection of Immune Serum

Rabbits injected in the foot pads and subdermally in the back with a suspension of virus in Freund's complete adjuvant were bled from the marginal ear vein 14 and 21 days after injection. The blood was

collected in a 50 ml centrifuge tube and allowed to clot at room
temperature. The clot was reamed, and retracted overnight at 4°C. The
serum was removed following centrifugation at 12,000 x g for 10 min
and heat-inactivated in a 56°C waterbath for 30 min. The serum was
sterilized by passage through a membrane filter (0.22 μm pore diameter)
(Gelman Instrument Co.), aliquoted and stored at -60°C.

Concentration of IgG Fraction of Rabbit Serum

Saturated ammonium sulfate (pH 7.0) was added slowly to an equal
amount of immune serum and precipitation allowed to continue overnight
at 4°C. The solution was centrifuged at 12,000 x g for 20 min to pellet
precipitated material and the supernatant discarded. The precipitate
was resuspended in one half its original volume of phosphate buffered
saline (PBS pH 7.4).

Lipoproteins were removed by dextran sulfate precipitation (Hudson
and Hay, 1976). To 25 mls of ammonium sulfate precipitated serum,
1.25 ml of 5% sodium dextran sulfate was mixed while stirring in an ice
bath. Calcium chloride (2.25 ml of a 1.0 M solution) was added and
the resulting precipitate was discarded after centrifugation at 12,000
x g for 10 min.

Rapid removal of salt was achieved by chromatography on a G-25
Sephadex column (LKB Inc.) with 0.25 M pH 9.0 carbonate-bicarbonate
buffer. The protein concentration of various fractions was determined
by optical density (O.D) measurements at 280 nm and then calculated by
the following formula: O.D. of 1.0 at 280 nm = 0.69 mg per ml of gamma
globulin.

Preparation of Fluorochrome Conjugate Immune Serum

The protein concentrations of the fractions representing IgG from rabbit anti-IPNV serum were adjusted to 20 mg per ml. Fluorescein isothiocyanate (Baltimore Biological Lab.) was added at a ratio of 0.05 mg per mg of IgG and the mixture stirred overnight at 4°C. The conjugated immunoglobulin was separated from free fluorochrome by passing the mixture over a G25 Sephadex column equilibrated with PBS (pH 7.4). The fractions representing conjugated IgG were pooled and stored at 4°C or frozen at -20°C.

Immunofluorescence

Direct and indirect fluorescent antibody techniques were used to determine the presence of viral antigens in persistently and lytically infected cells. These techniques have been described by Tu et al. (1974) and Piper et al. (1973).

Cells were prepared for immunofluorescent staining by seeding them onto glass coverslips (Gold Seal No. 0) in 60 mm petri dishes. Growth medium (MEM-10) was added and the cells were grown to monolayers (usually 3 days at 16°C). Lytically infected cells were prepared by infecting monolayers formed on the glass coverslips with 1000 $TCID_{50}$ of CL-IPNV and incubating for 24-36 h at 16°C. Persistently infected and uninfected control cells were prepared by seeding cells onto coverslips. The cells were incubated at 16°C until monolayers had formed. Cells on coverslips were washed in three changes of PBS and were fixed by immersion in cold acetone (-20°C). After a 10 min fixation, the coverslips were air dried at room temperature.

Fluorochrome labeled rabbit anti-IPNV 1:100 in PBS was added to each coverslip in direct fluorescent antibody tests. Fluorescein (FITC) conjugated rabbit anti-IPNV IgG was allowed to react with acetone fixed cells for 30 min at room temperature in a moist chamber. Unbound fluorochrome was removed by rinsing the coverslips in three changes of PBS.

Indirect fluorescent antibody tests were performed by reacting unlabeled rabbit anti-viral serum (1:100 in PBS) with acetone fixed cells for 30 min. Three washes in PBS removed excess unbound immunoglobulin. Fluorescein labeled goat anti-rabbit immunoglobulin (Baltimore Biological Lab.) diluted 1:50 in PBS was added to each coverslip. The coverslips were again incubated for 30 min at room temperature in a moist environment. Following the final incubation the cells were rinsed three times in PBS and air dried.

Stained and dried cells were prepared for microscopic observation by mounting coverslips onto microscope slides with buffered glycerol (pH 8.0) or Elvanol (polyvinyl alcohol pH 7.5). The cells were observed with a Zeiss fluorescent scope (Carl Zeiss Co.) illuminated with a 12V, 100W halogen or a 50 W mercury source. Filters (KP500 and KP490) were used for excitation in combination with LP560 and LP520 barrier filters. Photographs were taken with a Lietz 35mm camera and Tri-x pan or Ectachrome film (Eastman Kodak Co.).

Electron Microscopy

Persistently infected, lytically infected and uninfected STE-137 cells were examined by electron microscopy for the presence of IPNV and any ultrastructural differences. The cells were prepared for electron microscopy by fixing monolayers in situ with 4% glutaraldehyde buffered with Sorensen's PBS (0.2 m, pH 7.4). The monolayers were removed by scraping with a rubber policeman after a 2 h fixation at 16°C. Cells were removed following centrifugation at 2000 x g for 10 min, washed two times with PBS and stored at 4°C overnight. The cells were suspended in 2% ionagar (Difco Lab.) and after hardening were cut into 1 to 3 mm fragments. Agar embedded cells were postfixed and stained at room temperature with 1% (v/v) osmium tetraoxide (OsO_4) buffered with PBS (pH 7.4). The samples were dehydrated for 15 min at each change through a series of 30, 50 and 70% acetone in water solutions. During the dehydration, cells were stained with saturated uranyl acetate in 70% acetone. After dehydration was complete with changes of 85 and 100% acetone the cells were embedded in an Epon-Araldite mixture. The plastic embedding mixture was a modification of the Mollenhauer mixture No. 1 (Mollenhauer, 1959) and contained the following:

Dodecyl succinic anhydride – DDSA HY9643	53%
Araldite 6005	35%
Epon	12%
Benzyl dimethylamine – BDMA	0.05 ml/ml plastic

Noncatalyzed plastic was added to the cell pellets in a two to one ratio with acetone and left overnight at room temperature. Embedding

was completed by the addition of catalyzed plastic for 24 h at 60°C. Ultrathin sections were cut from cured plastic blocks with a Porter-Blum MT-1 ultramicrotome equipped with a diamond knife. Sections were mounted on 300 mesh grids and stained with lead citrate prior to viewing.

Negative stains of virus from lytically infected cells were prepared to determine virus size and morphology. Gradient purified virus suspensions were placed onto formvar coated grids and then mixed with a drop of 2% phosphotungstic acid (PTA) or 2% uranyl acetate. Excess stain was removed with filter paper and the samples were air dried. Negatively stained and thin sectioned preparations were examined with a Phillips EM300 transmission electron microscope at 60kV accelerating voltages. Micrographs were taken on Kodak electron imaging plates (Eastman Kodak Co.).

Homologous Virus Superinfection Test

Persistently infected CHSE-214 and STE-137 cells were tested for their ability to withstand challenges with homologous CL-IPNV. Persistently infected cells were grown in 25 cm^2 culture flasks with parallel sets of uninfected control cells. When both uninfected and persistently infected cells reached 90% confluency, duplicate samples of each were infected with selected dilutions of stock IPNV. Five ml aliquots of MEM-5 were added to all flasks and the cells were incubated at 16°C for 5 days. The number of cells in control flasks were counted at the time of challenge to determine the multiplicity of infection

by IPNV. The effect of homologous virus superinfection on the number of cells producing viral antigens as determined by immunofluorescent techniques was examined by preparing 10 coverslips with monolayers of persistently infected STE-137 cells. Cells on five of the coverslips were challenged with 1000 $TCID_{50}$ of Cl-IPNV, cells on the remaining coverslips received MEM-5 only. After 3 days all the cells on coverslips were fixed and stained for immunofluorescent microscopic examination.

Heterologous Virus Infection Test

Persistently infected (IPNV) CHSE-214 and STE-137 cells were challenged with IHNV, Herpesvirus salmonis and CSV to determine their relative susceptability to superinfection with these heterologous viruses. Uninfected and persistently infected CHSE-214 and STE-137 cells were grown in 25 cm^2 culture flasks. When the cells approached 90% confluency, flasks of persistently and uninfected cells (controls) were dispersed and counted to determine if approximately equal numbers were present. Flasks of cells were infected with 100 $TCID_{50}$ of one of the three challenge viruses. The virus was allowed to adsorb for 45 min then 5.0 ml of MEM-5 was added to each flask. The flasks were sealed, incubated at 16°C for 10 days and the cells observed for CPE. The culture fluids from cells challenged with IHNV were further examined to determine the difference in yields of IHNV between persistently infected and normal cells. Cellular debris was cleared by centrifugation at 2000 x g for 10 min. The clarified supernatants containing viruses were treated with anti-IPNV serum (1:50 dilution in MEM-10) for 1 h at 16°C. The yields of IHNV were then determined by $TCID_{50}$ analysis.

The plaquing efficiency of heterologous virus on persistently infected cells was determined by infecting persistently and uninfected CHSE-214 and STE-137 cells with IHNV. Cell monolayers in either multiwell dishes or 25 cm^2 flasks were infected with tenfold dilutions of IHNV as in the standard plaque assay. A methylcellulose plus 2X MEM-5 overlay was added following viral adsorption. The cells were incubated for 10 days at 16°C, then fixed in formaldehyde (37%), stained and the number of plaques counted. The plaquing efficiency of persistently infected cells was expressed as the percent reduction in the number of IHNV plaques on persistently infected versus control cells.

Interferon Determinations

Culture fluids from persistently infected cells were withdrawn prior to subculturing and tested for interferon activity against homologous and heterologous viruses. Methods for the detection of interferon from both fish serum (deKinkelin and Dorson, 1973) and from tissue culture fluids (deSena and Rio, 1975) have been reported. Culture fluids from PI cell lines were cleared of cellular debris and virus by centrifugation at 55,000 x g for 1 h. An aliquot was removed from the top of the ultracentrifuge tube and used immediately or stored at 4°C. Monolayers of CHSE-214 and STE-137 cells in 24 well dishes were treated with 0.1 ml aliquots of this centrifuged supernatant. The supernatant was dispersed over the monolayers by a rocking platform (Belco Co.) for 12-24 h at 16°C. Tenfold dilutions of Metolius River strain of IHNV, EVE or CL-IPNV were distributed to replicate wells of treated and untreated cells. The cells were overlayed with 2% methylcellulose or

1.6% agarose plus 2X MEM-5 following 45 min for virus adsorption. The cells were incubated at 16°C for 3 to 10 days at which time they were fixed and stained. Cells treated with the supernatant from uninfected cells processed in the same manner served as controls. In certain cases supernatants from persistently and uninfected cells were treated with anti-IPNV antiserum and subjected to the same procedure. Controls consisted of supernatant from uninfected cells which were treated with anti-IPNV antiserum. When anti-IPNV serum was added, Metolius River strain of IHNV was used as the only challenge virus.

Isolation of Uninfected Cells from the
Persistently Infected Cell Lines

Persistently infected cells were propagated in MEM-10 containing anti-IPNV serum to determine if uninfected cells could be derived from the persistent population. Persistently infected cells (CHSE-214/ CL-IPNV and STE-137/CL-IPNV) were dispersed with trypsin-EDTA and resuspended in a known volume of MEM-0. The cells were counted and then diluted in MEM-10 so that the highest dilutions would contain few or no cells. The cell dilutions were then transferred to multiwell dishes. Cells in six of twelve replicate wells were treated with anti-IPNV antiserum (1:50 in MEM-10). Cells in the remaining six wells received MEM-10 only. These dishes were sealed with a mylar film and observed daily for those wells containing the lowest number of cells in which growth and division occurred. After the cells had divided sufficiently to form a monolayer, the medium was removed and the cells were washed two times with MEM-10. Cells from antibody treated and

untreated wells were transferred to 25 cm^2 flasks for further propagation. After two passages in a 25 cm^2 flask, treated and untreated cells were challenged with freshly plaque-purified CL-IPNV.

Infectious Center Assay

The number of cells in the persistently infected population which could produce infectious IPNV was determined by the infectious center assay (Ahmed and Graham, 1977). Persistently infected cells were dispersed to single cells with trypsin-EDTA. The cells were centrifuged at 2000 x g for 5 min, resuspended in MEM-0 and centrifuged again. Following the second centrifugation, they were resuspended in 5.0 ml of MEM-0 containing a 1:100 dilution of anti-IPNV antiserum, incubated for 30 min and centrifuged a third time at 2000 x g. The supernatant was discarded and the pellet washed twice with MEM-0. The number of viable cells were counted with a hemocytometer after staining with trypan blue.

Tenfold dilutions were made when the number of viable cells were determined to be greater than 90%. Those preparations with viable cell counts lower than 90% were discarded. Dilutions (0.1 ml aliquots) of viable cells were plated into 25 cm^2 flasks and 5 x 10^6 uninfected (CHSE-214) cells in MEM-10 were added and mixed gently with persistently infected cells. After all the cells had firmly attached to the surface of the flask, (approximately 3 h) an agar overlay containing equal parts of 1.6% ionagar and 2X MEM-5 was added. Duplicate flasks of each cell dilution were fixed and stained after 5 days incubation at 16°C. The number of plaques and the number of cells distributed to each flask

was determined. The quantity of infectious centers was calculated by dividing the number of plaques at a given dilution by the total number of cells from the persistently infected population distributed at that dilution. This value is expressed as the percentage of infectious centers.

Purification of Viruses from Lytically Infected Cells

Viruses were recovered from lytically infected cells by one of two purification methods. The initial purification was a modification of that described by Cohen et al. (1973). The second method was that of Chang et al. (1978).

Viral nucleic acids were labeled with either ^{32}-P orthophosphate or 5-^{3}H-uridine (ICN Radiochemicals) at 10 µCi and 2 µCi/ml respectively. Cells were grown in 75 or 150 cm^2 flasks (Corning Glass Works) and when 90% confluency was reached, the medium was removed and replaced with 2.0 ml of stock CL-IPNV. The multiplicity of infection ranged from 10-20 TCID$_{50}$ per cell. The viruses were adsorbed over a 2 h period at 16°C. Fresh MEM-5 with ^{3}H-uridine or Ham's F-10 (Grand Island Biological Co.) medium without phosphate with 5% fetal calf serum and ^{32}-P orthophosphate was added to each flask. The culture flasks were sealed and incubated at 16°C for 3 to 5 days at which time the cells exhibited maximum CPE.

Cells were removed by scraping with a rubber policeman and pooled with the culture fluid. Virus preparations purified by the method of Cohen et al. (1973) were sonicated two times for 30 sec with a sonifier

(Heat Systems – Ultrasonics Inc.) at the maximum setting of the micro-tip in an ice bath. The sonicated material was centrifuged at 55,000 x g for 2 h and the resulting pellet resuspended in 1.0 ml of TNE buffer. An equal volume of cold Genetron[R] (trifluorotrichloroethane, Allied Chemical Co.) was added to the solution which was then placed on a vortex mixer for 5 min at maximum speed with intermittent cooling in an ice bath. The upper aqueous phase was collected after centrifugation at 12,000 x g for 10 min in a SS-34 rotor (Ivan Sorvall Inc.). After a second Genetron[R] extraction, the upper phase was layered over a 20% (w/v) sucrose column and centrifuged at 114,000 x g for 1 h. The virus pellet was resuspended in 1.0 ml of TNE buffer. Cesium chloride (CsCl) was added to the virus suspension to attain a density of 1.30 g/cm^3. The virus was concentrated into a band by centrifuging at 114,000 x g in a SW 50.1 rotor (Beckman Instruments Inc.) for 18 h at 5°C.

Virus preparations purified by a modified method of Chang et al. (1978) were found to be more satisfactory. Cells and supernatant were harvested in the same manner already indicated and then separated by centrifugation at 5000 x g in a GSA rotor (Ivan Sorvall Inc.) for 15 min. The supernatant was collected and NaCl added to a concentration of 2.2% while stirring. Polyethylene glycol MW 6000 (J.T Baker Chemical Co.) was mixed to a concentration of 10%. Precipitation proceeded with constant stirring for a minimum of 4 h but usually for 16 h at 4°C. This precipitate was collected by centrifugation for 1 h at 10,000 x g in a GSA rotor and the virus containing pellet resuspended in 2.0 ml of TNE buffer.

The cellular fraction separated in the first step was resuspended in 2.0 ml of TNE buffer and subjected to a Genetron^R extraction as indicated previously. The aqueous phases from the cellular and supernatant fractions were layered over a stepwise gradient of CsCl (1.5 ml 40%, 1.0 ml 30%, 0.5 ml 20%). Mineral oil was added to the top of the nitrocellulose ultracentrifuge tube and the preparation centrifuged at 114,000 x g for 18 h in a SW 50.1 rotor. Bands containing virus were collected by fractionation with a Densiflow (Buchler Instruments Inc.) or by side puncture with a 23 gauge needle fitted on a 5.0 ml syringe. In certain cases bands containing virus were dialyzed against TNE buffer for 1 h at 4°C then layered over a second stepwise CsCl gradient and centrifuged at 114,000 x g for 5 h. The virus containing bands were again collected and dialyzed in TNE at 4°C.

Purification of Viruses from Persistently Infected Cells

Virus was purified from persistently infected cells and supernatant to determine differences from virus obtained from lytically infected cells. Persistently infected cells were labeled with 5-^{3}H-uridine at levels between 5 and 20 uCi/ml of MEM-5. As persistently infected cell monolayers approached 50 to 70% confluency, old growth medium was replaced with medium containing ^{3}H-uridine. When 100% confluency was reached, the cells and supernatant were harvested and the viruses were purified by the same procedures described for lytically infected cells. Because virus concentrations were insufficient to form visible bands, entire gradients were collected by fractionation into 200 μl aliquots. These fractions were analyzed for trichloracetic

acid precipitable radioactivity. The infectivity and interfering capacity of various fractions were also determined after dialysis against TNE buffer at 4°C.

Bouyant Density Determinations

The densities of viruses in fractions collected from CsCl gradients were determined by reading their refractive indices with a refracto-meter (Bausch and Lomb Inc.). A correction factor of 0.0015 was subtracted from each refractive index. This value represented the difference between double distilled water and TNE buffer which was the solvent for CsCl gradients. The densities were determined by conversion using the International Critical Tables.

Infectivity and Interfering Capacities of Virus
Separated by CsCl Gradients

The infectivity and interfering capabilities of viruses isolated by means of CsCl gradients were tested by $TCID_{50}$ analysis. Fractions representing defective and infective viruses as determined by radio-active labeling were dialyzed against TNE to remove CsCl. The fractions were then added to an antibiotic solution (500 units penicillin, 500 mcg streptomycin, gentamycin 250 mcg and 100 units mycostatin per ml) and incubated for 24 h at 4°C. The determination of interfering capabilities was tested by making tenfold dilutions of selected fractions and distributing 0.5 ml aliquots to monolayers of STE-137 cells grown in 25 cm^2 or multi-well dishes. Cells were pretreated in this manner for 12 h prior to challenge with purified or stock

infectious virus. Replicate wells of treated and untreated STE-137 cells were titered following infectious virus challenge for yields of IPNV by $TCID_{50}$ analysis.

Establishment and Care of Carrier Brook Trout

Brook trout approximately 3.5 g in weight were obtained from Fall River Hatchery (Oregon Department of Fish and Wildlife). Fish were held in 68 L fiberglass tanks receiving single pass, fish pathogen-free well water flowing at a volume of 3.8 L per min. Fourteen fish distinguishable by individual cold brands were held in a single tank at 12°C.

Carriers were established by feeding the young brook trout Oregon Moist Pellet (OMP) ratio containing CL-IPNV at $10^{4.5}$ $TCID_{50}$/g for 2 weeks. Ration without virus was fed for 8 additional weeks and then fecal samples taken at weekly intervals for 10 weeks. During the period when fecal samples were collected, carriers received regular OMP once daily.

Titration of Virus in Carrier Brook Trout Feces

The carrier status of individual brook trout was monitored by examining fecal samples collected at weekly intervals for viral content. Carrier trout were netted and transferred to a container with 5 L of well water containing 1.5 ml of 10% benzocaine for anesthesia. Feces from anesthetized trout were expressed by gently compressing the lateral surfaces with the hand. Feces were collected in 1.5 ml centrifuge tubes (Pacific Coast Biological Co.) and the total weight of feces per tube

recorded. An antibiotic solution was added to bring the feces to a
1:10 dilution (w/v). The fecal solution was blended vigorously with a
vortex mixer to break up the larger aggregates. The samples were
incubated at 4°C overnight to prevent microbial contamination. At the
end of the incubation period the samples were again blended and
then centrifuged at 2000 x g for 10 min to sediment particulates.
The supernatant was tested for infectivity by $TCID_{50}$ and the virus titer
recorded as the number of $TCID_{50}$ per gram of feces. The lower limit of
viral detection of these titrations was $10^{1.6}$ $TCID_{50}$ per gram. The
lower limit resulted from the tenfold dilution of fecal material prior
to application to cell culture. Therefore if CPE occurred at this
lowest dilution the minimum titer was $10^{1.6}$ $TCID_{50}$ per gram.

Titration of Virus in Carrier Brook Trout Tissues

At the end of the ten week fecal sampling period, all carrier trout
were sacrificed and selected internal organs analyzed for levels of IPNV.
Portions of the kidney, spleen and caeca were aseptically removed and
placed into sterile tared 12 x 75 mm tubes. A solution containing
antibiotics at concentrations previously mentioned was added to each
tube to make a 1:10 dilution (w/v) of the tissue sample. Tissue
preparations were disrupted by sonication during two separate intervals
of 5 sec each with the microtip at maximum setting (Heat Systems -
Ultrasonics Inc.). Cellular debris was removed by centrifugation at
5000 x g for 10 min and the supernatants stored at 4°C overnight.
These supernatants were tested for infectivity by $TCID_{50}$ analysis.

Determination of Anti-IPNV Neutralizing Titers of
Carrier Brook Trout Serum

The serum from sacrificed brook trout carriers was titered to determine if levels of anti-IPNV neutralizing antibodies could be correlated with concentrations of virus found in the feces and selected internal organs. Blood was collected in 1.5 ml centrifuge tubes after severing the caudel artery. The blood was allowed to clot at room temperature and then centrifuged for 30 sec in a microfuge (Beckman Instruments Inc.). The serum was collected and sterilized by passage through a membrane filter (0.22 μm pore diameter) and stored at -20°C. Twofold dilutions of serum were prepared in MEM-0 and an equal amount of MEM-0 containing $10^{2.4}$ $TCID_{50}$ of CL-IPNV was added to each tube. The suspension was agitated at 15 min intervals during the 1 h incubation at 16°C. Aliquots (0.1 ml) from each tube were transferred to individual wells (multi-well dish) and one drop of a suspension of CHSE-214 cells (approximately 2×10^6 cells per ml) in MEM-10 was added. The multi-well dishes were sealed and incubated at 16°C for 7 days. The neutralizing titer was determined for each serum sample by calculating the dilution of serum at which 50% of the replicate wells had neutralized the virus and were free of CPE.

Infectious Center Assays (ICA) of Carrier Brook Trout Tissues

The number of cells in the kidney of carrier brook trout that could act as infectious centers was calculated to determine similarities with persistently infected cell cultures. Kidney tissues from carrier

trout were aseptically removed and placed into 60 mm petri dishes

(Falco Plastics) containing MEM-O. The tissues were rinsed by a series

of three MEM-O changes. Two scalpels were used to mince the tissue into

fragments of approximately 0.5 mm^3. These fragments were rinsed again

and then placed into a screw cap tube containing 0.25% trypsin in

MEM-O. Trypsinization continued for 30 min with periodic stirring.

The trypsin solution was removed by centrifugation at 2000 x g for

5 min and the supernatant discarded. Excess virus and trypsin were

removed by two rinses in MEM-O followed by centrifugation (2000 x g).

Anti-IPNV antiserum diluted 1:100 in MEM-O was combined with the cells

after the second wash and the mixture incubated for 30 min at 16°C.

The remainder of the assay was identical to the method previously

described for persistently infected cell cultures.

Electron Microscopy of Carrier Brook Trout Tissues

Electron microscopy was employed to compare the ultrastructure of

carrier trout tissues to persistently infected cell cultures. Kidney,

spleen, caeca and feces from representative carrier trout were cut into

fragments approximately 1 mm and placed into Hawke's fixative (89.0%

0.1 M cacodylate buffer, 1.5% paraformaldehyde, 0.5% acrolein and 0.75%

glutaraldehyde). Tissues were fixed for 2 h at 16°C at which time the

fixative was replaced with 0.1 M cacodylate buffer. Post fixation,

embedding and staining of tissue samples were identical to the procedure

for cultured cells.

EXPERIMENTAL RESULTS

Establishment of Persistently Infected Cell Lines

Both continuous cell lines (CHSE-214 and STE-137) are susceptable
to IPNV producing severe CPE in two to three days at 16°C. Super-
natants harvested from IPNV lytically infected cell lines contains $10^{7.5}$
to $10^{9.0}$ $TCID_{50}$/ml of infectious virus. Cells surviving the initial
CPE may continue to replicate and form a new monolayer.

The persistently infected (PI) STE-137 cell line arose from a small
population of cells surviving an initial infection with CL-IPNV.
Following a period of maximum CPE (3 days), the culture fluid was
removed and replaced with fresh MEM-10. Small groups of cells became
apparent after 3 weeks incubation at 16°C. After 5 more weeks of
incubation these cells had divided sufficiently to form a complete
monolayer. This PI cell line was designated STE-137/CL-IPNV. The
morphology and growth characteristics of these cells were indis-
tinguishable from normal uninfected cells. No CPE was observed at any
passage level of the STE-137/CL-IPNV cell line.

Serial passages of IPNV at high MOI, a procedure known to encourage
the production of defective interfering viruses with rhabdoviruses and
reoviruses (Palma and Huang, 1974 and Ahmed and Graham, 1977),
increases the number of cells that will survive a lytic IPNV infection
(Nicholson and Dunn, 1974). Serial undiluted passages of CL-IPNV were
therefore undertaken to determine if persistence could more rapidly
be established with virus inocula that contained substantial numbers of

defective interfering viruses. The PI CHSE-214/CL-IPNV cell line was

established by infecting CHSE-214 cells with CL-IPNV which had been

passed three times at an MOI of 100-1000 $TCID_{50}$ per cell. A large

number of cells survived the initial challenge and within a week had

established a monolayer. The cells at this stage were of abnormal

morphology and unstable upon subculture, exhibiting CPE with large

numbers of the cell population perishing. Surviving cells would then

grow and divide to form new monolayers. Following a period of

instability (crisis) in early passages, the cells became morphologically

indistinguishable from normal CHSE-214 cells and were stable to

subculturing. At higher passage levels small foci of CPE were

occasionally observed in the monolayer. However, these areas never

became widespread and were replaced by normal appearing cells soon

after they were formed.

Characteristics of Persistently Infected Cell Lines

Release of Infectious Virus

The persistence of IPNV was monitored by titrating the amount of

infectious virus released into the culture fluids of both PI cell

lines at each weekly subculture. The concentrations of CL-IPNV released

from both PI cell lines fluctuated from one passage to the next

(Figure 1).

Infectious virus was still being released after 29 passages of

the PI STE-137 cell line. Peak titers in this culture were reached
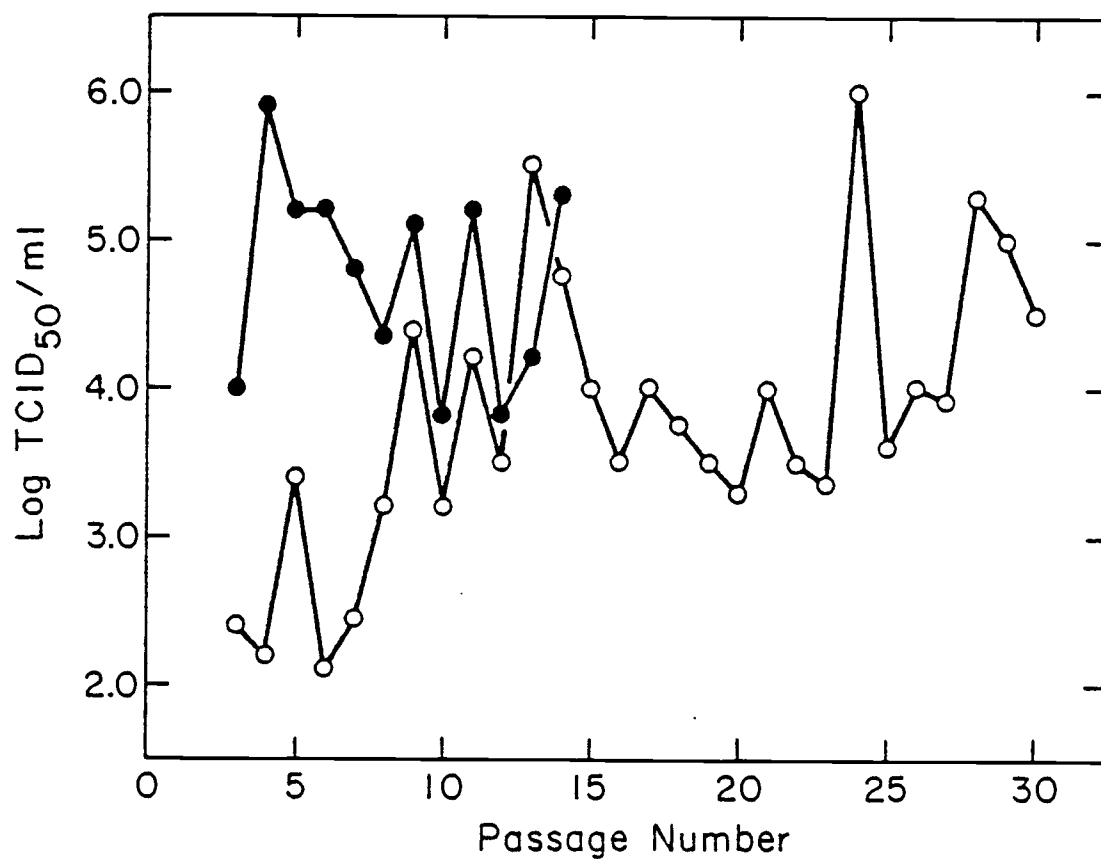
Figure 1. The concentrations of infectious virus detected in the
supernatant fluids of STE-137 (0-0) and CHSE-214 (●-●)
cell lines persistently infected with infectious
pancreatic necrosis virus (CL-IPNV).

at passage numbers 14 and 24 when the concentrations of infectious

virus rose to $10^{5.5}$ to $10^{6.0}$ $TCID_{50}$/ml between consecutive passages.

The PI CHSE-214 cell line exhibited the greatest variation in

infectious virus concentrations between passages. During the initial

passages, cells would exhibit CPE upon subculturing and the viral titers

of the culture fluid were relatively high ($10^{6.0}$ $TCID_{50}$/ml). By the

fourth passage there were reduced yields and less variation in the

concentrations of infectious virus released.

The continued release of low concentrations of infectious virus in

the absence of CPE indicated that viral persistence was a stable

relationship between the cell lines and CL-IPNV.

Light Microscopy

Microscopic examinations of PI cell lines demonstrated that the

production of infectious virus can proceed without observable CPE. Both

PI cell lines (Figure 2c and d) were indistinguishable from control

uninfected cells (Figure 2a and b). The only exceptions were the

observation of CPE during early passages and an occassional foci in later

passages in PI CHSE-214 cells. These foci were rare and were rapidly

replaced by normal appearing cells.

Cells lytically infected with CL-IPNV at low MOI (Figure 2g and h)

and high MOI (Figure 2e and f) demonstrated characteristic IPNV CPE

after 48 h. The CPE was more extensive in lytically infected cells

at lower MOI compared to cells infected with CL-IPNV that had been

repeatedly passed at high MOI (Figure 2e and f). Defective inter-

fering virus produced by serial undiluted passages of CL-IPNV was

Figure 2. Phase contrast photomicrographs of (a) CHSE-214 and (b)
STE-137 cell lines which are uninfected; (c) CHSE-214
and (d) STE-137 cell lines which are persistently infected;
(e) CHSE-214 and (f) STE-137 cell lines lytically infected
at high MOI (100); (g) CHSE-214 and (h) STE-137 cell lines
lytically infected at low MOI (0.01) with infectious
pancreatic necrosis virus (CL-IPNV).
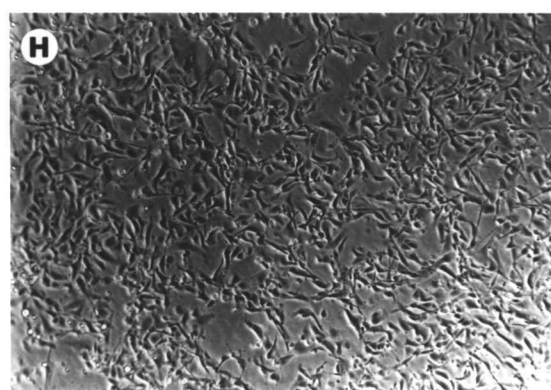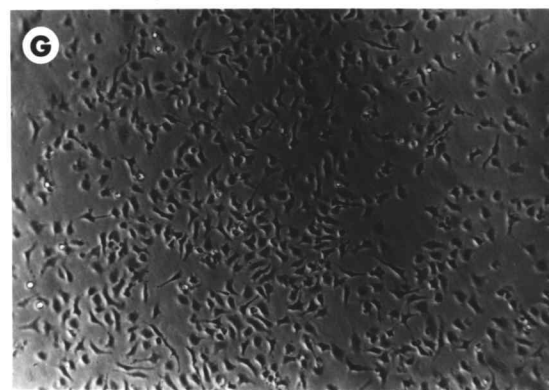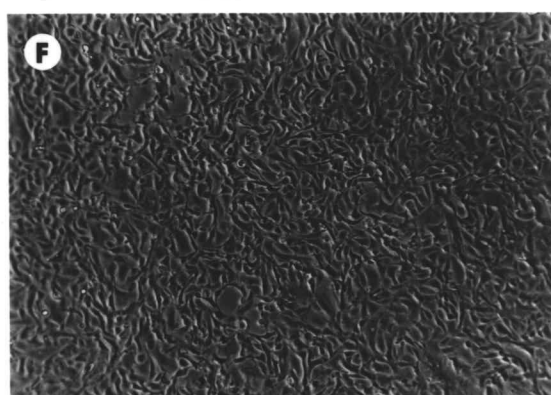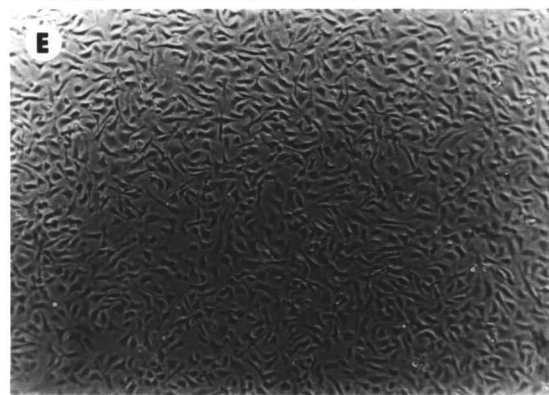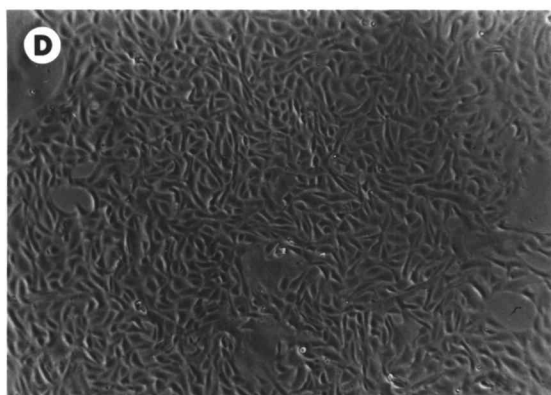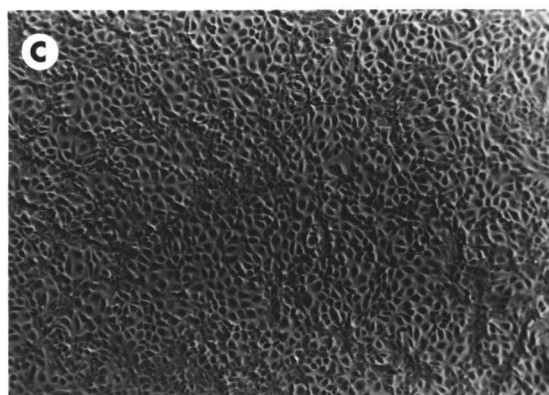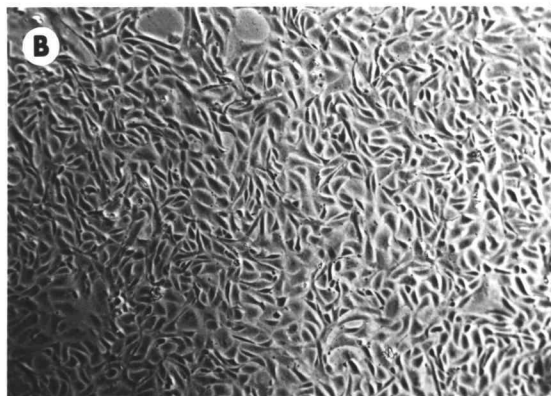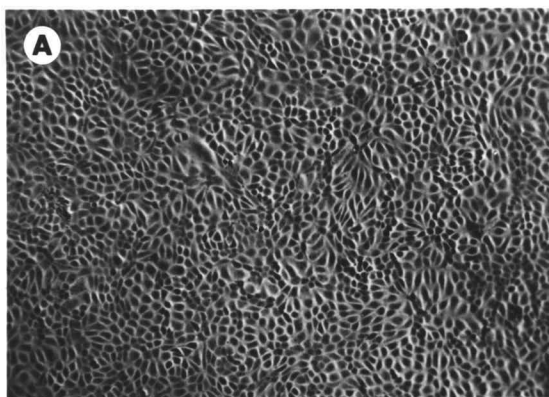
probably responsible for decreasing the CPE in CHSE-214 and STE-137

cells infected at high MOI.

Persistently infected cell lines retained a similar morphology and

growth rate to uninfected cells throughout this study. The production

of infectious virus that is released by PI cell lines proceeds most

often in the absence of observable CPE.

Immunofluorescence

Fluorescent antibody tests were used to determine if the cells in

PI lines that were morphologically indistinguishable by light microscopy

from uninfected cells, might be producing virus or viral antigens. These

tests demonstrated that only a small number (1% or less) of the cells in

the population are producing detectable levels of IPNV antigens (Figure

3c and d). Viral antigens were always confined to the cytoplasm of

these cells, where either small spots of fluorescence or more commonly

the entire cytoplasm was brightly stained. The condition of brightly

stained cells was often similar to those observed in advanced stages

of lytic infections (Figure 3a and b). Similar staining patterns have

been observed by Tu et al. (1974) and Piper et al. (1973) in lytically

infected RTG-2 cells.

The results of immunofluorescent tests demonstrated that similar

staining patterns may be observed in lytic and persistent infections

but the number of cells participating in viral replication is greatly

reduced in the latter. The occasional death of such a small percentage

(1% or less) of the cells would explain the difficulty in observing

CPE by light microscopy.

Figure 3. Photomicrographs of immunofluorescent stained lytically infected (a) STE-137 and (b) CHSE-214 cell lines and persistently infected (c) STE-137 and (d) CHSE-214 cell lines with infectious pancreatic necrosis virus (CL-IPNV).
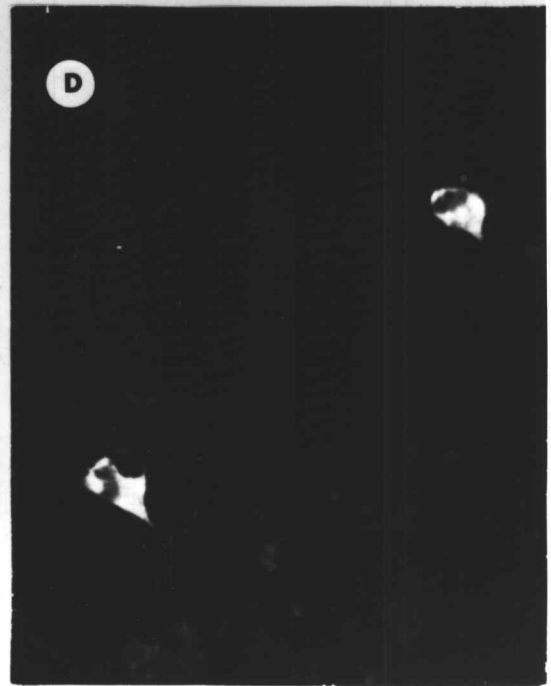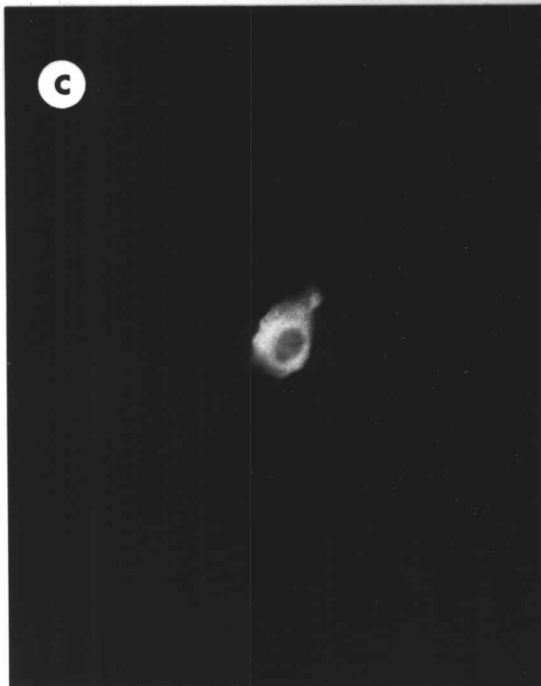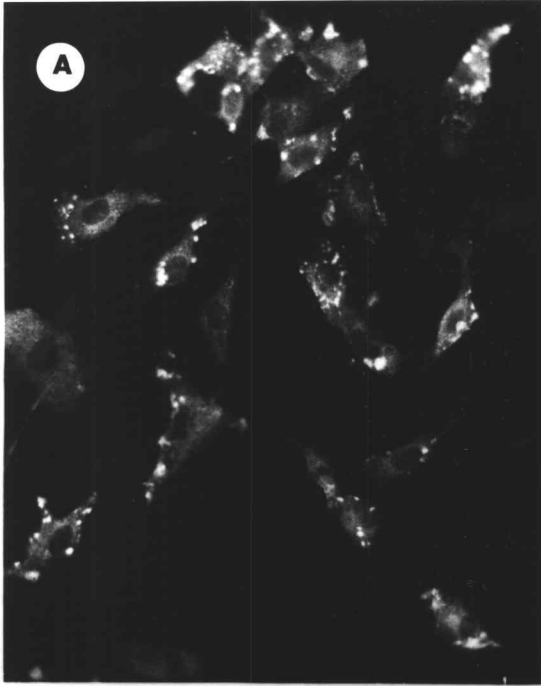
Infectious Center Assays

Immunofluorescent tests indicated that only a fraction of the cells were producing viral antigens in the PI cell population. Fluorescent antibody tests detect viral antigens but cannot determine if cells are actually releasing infectious virus. Infectious center assays were used to enumerate the number of cells in the population producing infectious virus. Infectious center assays indicated that 1% or less of the cells in PI cell lines were producing infectious virus (Table 3). Two assays were made with each PI cell line to determine if the percent of infectious centers might fluctuate between passages. In all cases the percentage of cells acting as infectious centers was between 0.1 and 1.0%. The majority of the cells in the PI cell population do not act as infectious centers and are probably not involved in infectious virus production.

Isolation of Uninfected Cells from Persistently Infected Cell Lines

Infectious center assays demonstrated that only 1% or less of the cells in PI cell lines are producing infectious virus. Propagations of PI cell lines in medium containing antibody were performed to determine if preventing the diffusion of virus from producing cells to others in the population would disrupt persistence and allow the selection of uninfected cells. Cloning of PI cell lines was not practical because of the very low efficiency of plating of these cells. The same problem was encountered when attempts were made to clone un-infected CHSE-214 and STE-137 cells.

Table 3. Percent infected cells at selected passage numbers of cell lines persistently infected with infectious pancreatic necrosis virus as determined by infectious center assays.

Cell Line	Passage No.	Viable Cell Count	Infectious Centers
STE-137/CL-IPNV	P21	2.3×10^5	0.85^a
	P25	1.0×10^6	0.33
CHSE-214/CL-IPNV	P1	1.5×10^6	0.1
	P7	5.8×10^6	1.0

[a]Expressed as the percent of the total number of cells acting as infectious centers.

In place of cloning, groups of PI cells at the lowest concentration allowing survival (approximately 10^2 cells/ml) were propagated in MEM-10 containing anti-IPNV antibody ($10^{5.0}$ ND_{50}/ml). Groups of uninfected cells were isolated from PI cell lines by seven such passages in medium containing antibody (anti-IPNV). After two passes without antibody these cells were susceptable to 100 $TCID_{50}$ challenges with stock CL-IPNV. Persistently infected cells propagated in an identical manner but without antivirus antibody remained persistently infected as demonstrated by their resistance to 100 $TCID_{50}$ challenges with CL-IPNV and their continued production of virus.

In three experiments, antibody was removed prior to a sufficient number of cell passages, these cells experienced extensive CPE. This indicated that curing of persistent infection was not a rapid process but instead involved the progressive removal of virus and virus producing cells at each subculture.

Resistance to Superinfection with Homologous Virus

Infectious virus was continually present in the culture fluids of persistently infected cell lines as determined by weekly titrations. Immunofluorescent and infectious center assays further demonstrated that only 1% or less of the cells were producing viral antigens, the remaining 99% were not. Persistently infected cell lines were therefore challenged at high MOI with both CL-IPNV and several serologically diverse IPNV strains to determine (1) if the protection of a majority of the cells could be overwhelmed by an excess of infectious virus and

(2) if protection extended to serologically diverse IPNV strains. The PI STE-137 cell line was resistant to superinfections with CL-IPNV, VR-299 and EVE at MOI of 1000. Both cell lines were also resistant to low MOI (0.01) challenges with CL-IPNV, Buhl-IPNV, CTT-IPNV and EVE. In both studies control cells infected with these viruses were completely destroyed after 3 days.

The number of cells in the PI STE-137 cell line as passage number 29 that demonstrate viral antigens by immunofluorescent staining was not changed by superinfection with CL-IPNV at an MOI of 100, a level sufficient to infect 100% of control STE-137 cells. These results indicated that the protection of a majority of the cells in PI lines (1) is effective in the presence of high levels of infectious virus and (2) extends to serologically diverse strains of IPNV.

Susceptability to Infection with Heterologous Viruses

Persistently infected cells were challenged with three heterologous viruses to determine (1) if the protection provided to homologous virus extended to heterologous viruses and (2) how well heterologous viruses replicated in PI compared to control cells. Both PI cell lines were susceptable to 100 $TCID_{50}$ challenges with three heterologous fish viruses. Herpesvirus salmonis, chum salmon virus (CSV) and Metolius River strain of IHNV all caused characteristic viral CPE.

The plaquing efficiency of Metolius River strain of IHNV was slightly reduced in the PI STE-137 line compared to control cells (Table 4). The PI CHSE-214 cell line showed little reduction compared

Table 4. Comparison of the plaquing efficiency of
          Metolius River strain of infectious
          hematopoietic necrosis virus on normal
          and persistently infected cell lines
          with infectious pancreatic necrosis virus.

Cell Line	Titer of IHNV
CHSE-214	8.0×10^{6a}
CHSE-214/CL-IPNV	7.5×10^{6}
STE-137	6.5×10^{6}
STE-137/CL-IPNV	1.7×10^{5}

[a]Expressed as PFU per ml.

to control cells (Table 4). The slight differences in plaquing efficiencies between PI and control cells was not reflected in the yields of Metolius River strain of IHNV from lytic infection in these cell lines. Comparable yields of IHNV were obtained from both control and PI cell lines (Table 5). The replication of heterologous viruses in these cell lines does not seem to be influenced as a result of persistent infection with CL-IPNV and suggested that the factor(s) responsible for resistance to superinfection by PI cell lines was specific for homologous virus.

Nature of Viral Persistence

Determinations of Interferon

The susceptibility of PI cell lines to heterologous virus infections suggested that interferon which has a broad anti-viral activity was not important in controlling virus replication. This was further demonstrated by assays performed to detect the presence of interferon in culture fluids from both PI cell lines.

An assay used to determine the presence of interferon in rainbow trout sera (deKinklen and Dorson, 1975) and in tissue culture fluids from IPNV infected RTG-2 cells (deSena and Rio, 1975) failed to detect activity in culture fluids of either cell line persistently infected with CL-IPNV or uninfected control cells (Table 6). If interferon was present in the culture fluids there should have been reductions as great as 5000 fold (deSena and Rio, 1975) in the plaque titer of IHNV between experimental and control cells. There were slight reductions in both

Table 5. Comparison of the yields of Metolius River strain of
infectious hematopoietic necrosis virus from normal
and persistently infected cell lines with infectious
pancreatic necrosis virus.

Cell Line	Passage No.	Titer IHNV[a]	
		Normal	Persistently Infected
CHSE-214/CL-IPNV	7	$10^{7.0}$	$10^{6.6}$
	15	$10^{7.2}$	$10^{7.1}$
	24	$10^{6.7}$	$10^{7.3}$
STE-137/CL-IPNV	26	$10^{6.5}$	$10^{6.3}$
	44	$10^{7.3}$	$10^{7.0}$

[a]Expressed as the $TCID_{50}$ units per ml of supernatant fluid.

Table 6. Results of determinations to detect interferon in the
culture fluid of normal and cell lines persistently
infected with infectious pancreatic necrosis virus
by reducing the plaque titer of Metolius River strain
of infectious hematopoietic necrosis virus.[a]

Source of Culture Fluid	IHNV Plaque Titer
STE-137/CL-IPNV	7.0×10^5 [b]
STE-137/ Normal	3.0×10^6
CHSE-214/CL-IPNV	1.5×10^6
CHSE-214 Normal	4.0×10^6

[a]Control CHSE-214 and STE-137 cells were treated with culture
fluids from persistently infected or uninfected cell lines for
12-24 h and then challenged with IHNV.

[b]Expressed as PFU per ml.

cell lines but these were less than expected if appreciable amounts of interferon were present.

Effects of Temperature

Cell lines persistently infected with reovirus (Graham, 1977) and VSV (Younger et al., 1976) which are propagated at temperatures below the optimum for virus replication undergo crisis periods when shifted to a more favorable temperature. These crisis periods are characterized by CPE and increased releases of infectious virus. The optimal temperature for IPNV replication is 20-22°C (Malsberger and Cerini, 1965). When persistently infected cell lines routinely propagated at 16°C were shifted to 22°C there were no indications of crisis, instead both cell lines grew more rapidly at the elevated temperature (Table 7).

The concentrations of infectious virus produced by PI cells held at 16°C and those shifted to 22°C were compared to determine if the production of infectious virus was influenced by a temperature change. The concentrations of infectious virus released by cells propagated at both temperatures were approximately the same (Table 7).

The effect of temperature on PI cell lines was further examined by testing the temperature sensitivity of the virus released following propagation at 22°C. Virus released from both PI cell lines exhibited approximately the same temperature sensitivity (^{22}/16°C = .02 - .32) regardless of the temperature at which the cells were incubated (Table 7).

Table 7. Titers of infectious virus released into the culture fluids from cell lines persistently infected with infectious pancreatic necrosis virus following propagation at 16 and 22°C for 9 days.

Cell Line	Passage No.	Cell Numbers	Temp °C Cells Propagated	Temp °C of Assay	Titer[a]	22/16°C[b] ratio
STE-137/ CL-IPNV	P41	6.6×10^5	16	16	$10^{4.6}$	.03
				22	$10^{3.1}$	
		8.3×10^5	22	16	$10^{4.3}$	.02
				22	$10^{2.6}$	
CHSE-214/ CL-IPNV	P21	6.7×10^5	16	16	$10^{3.9}$	.32
				22	$10^{3.1}$	
		1.8×10^6	22	16	$10^{3.6}$	.03
				22	$10^{2.1}$	
CL-IPNV[c] Control		1.2×10^6	16	16	$10^{7.5}$	.41
				22	$10^{7.1}$	
		2.5×10^6	22	16	$10^{7.7}$	.79
				22	$10^{7.6}$	

[a] Standardized to $TCID_{50}$ per 10^6 cells. Each value represents the geometric mean of duplicate titrations.

[b] Expressed as the ratio of virus titer at 22 compared with 16°C.

[c] Titers of infectious virus released into the culture fluids were determined after 3 days.

The virus released by both PI cell lines was slightly more sensitive (20 times) to 22° than stock wild type CL-IPNV (the virus used to establish PI cell lines). The production of temperature sensitive (ts) virus in cell lines persistently infected with VSV is known to be important in controlling infectious virus replication (Youngner et al., 1976). Virus from culture fluids of these cell lines are 10,000 fold more sensitive to elevated temperatures than wild type virus. Results of the temperature experiments in this study indicate that virus production in cell lines persistently infected with CL-IPNV is not a function of the temperature of incubation. In addition, the temperature sensitivity of the virus released suggests that factor(s) other than ts viral mutants are important in controlling and maintaining viral persistence.

Electron Microscopy

The first indication that an important factor in viral persistence might be the production of defective virus came from an electron microscopic examination of PI STE-137 cells at passage number 6. Certain cells in PI cultures were found to contain many structures approximately 55-57 nm in diameter in the cytoplasm (Figure 4d). In some cases these particles were clearly identified as complete virions (Figure 4b) but more often were less electron dense (Figure 4f). Incomplete virions were usually contained in membrane vacuoles and in greater numbers than complete virions. In contrast, cells lytically infected with CL-IPNV contained large numbers of complete virions and and very few structures resembling incomplete virions (Figure 4c and e).

Figure 4. Electron micrographs of the STE-137 cell lines (a)
uninfected, (c) (e) lytically infected and (b) (d)
(f) persistently infected with infectious pancreatic
necrosis virus (CL-IPNV). Arrows indicate defective
or infective virions which had a mean diameter of
55 nm.
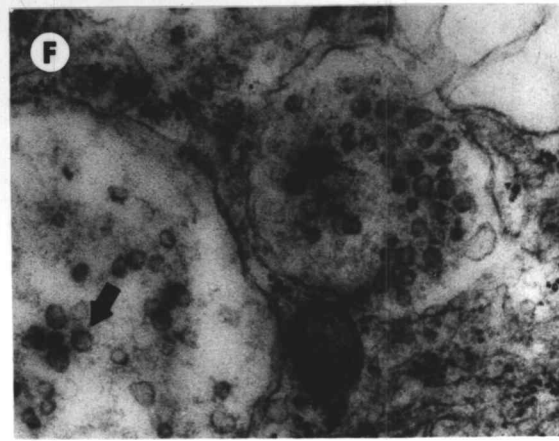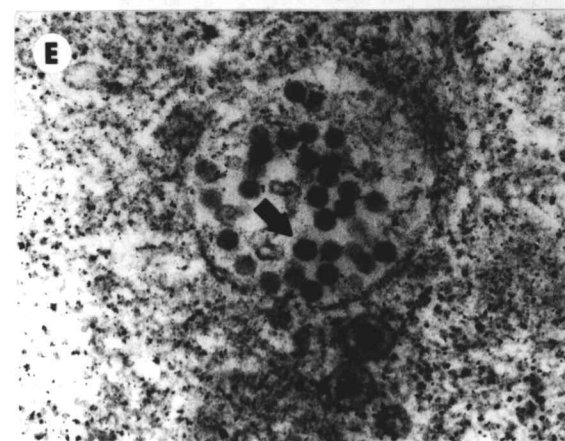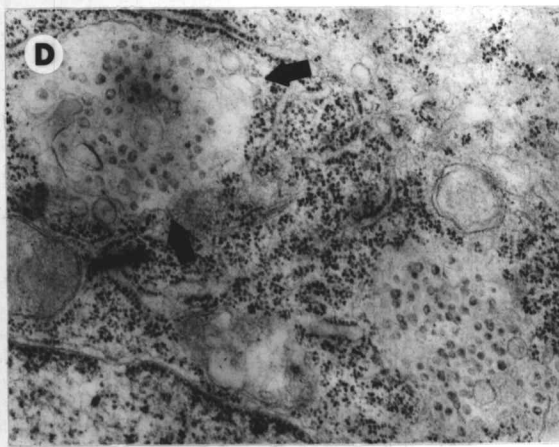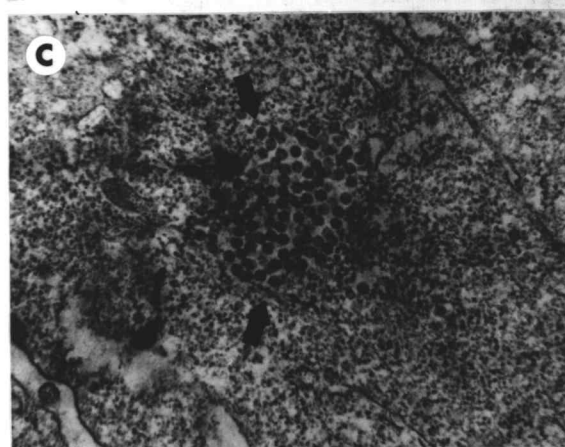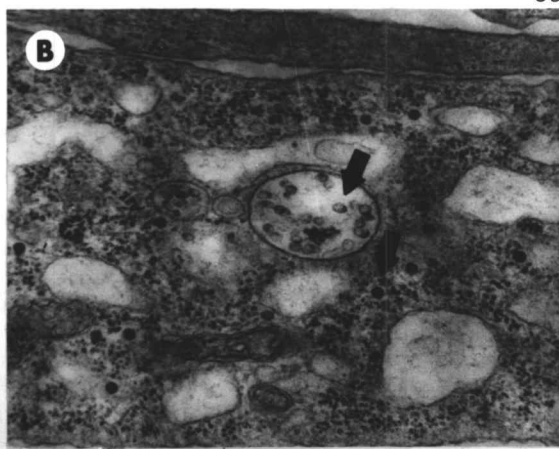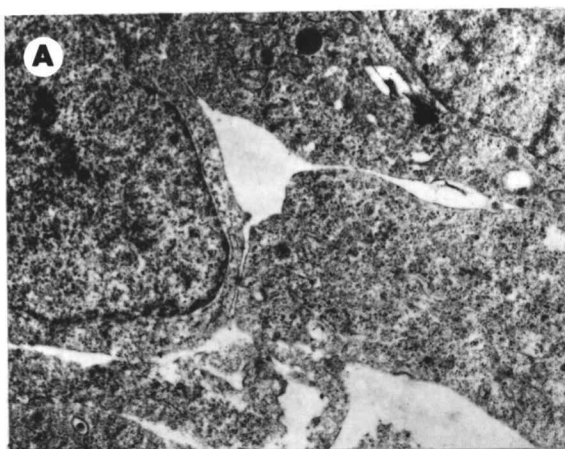
Uninfected cells did not contain structures resembling either complete or incomplete virions (Figure 4a). Complete virions isolated from lytically infected CHSE-214 cells are shown in Figure 5. The diameter of virions in this hydrated state was 70 nm.

Defective Interfering Virus

Electron microscopic studies demonstrated that the production of virus in PI cells was characterized by the presence of defective virions which are seldom seen in lytically infected cells. The production of virus was further examined by radioisotope techniques designed to label viral RNA. Virus was then extracted from lytically and persistently infected cells and isolated by isopycnic centrifugation in CsCl.

The incorporation of $5-^3H$ uridine into viral RNA was markedly different between PI and lytically infected cell cultures. In lytically infected cell cultures ^3H-uridine was incorporated into particles that banded at a density of 1.33 g/cc, the density of complete infectious virus (Figure 6). Lower levels of incorporation were observed into particles with a density of 1.29 g/cc and 1.26 g/cc.

Persistently infected STE-137 cultures were characterized by high levels of ^3H-uridine incorporation into particles that banded at a density of 1.29 g/cc with less at 1.33 and 1.26 g/cc (Figure 7). The infectivity was associated with the virus band of 1.33 g/cc. Particles with a density of 1.29 g/cc exhibited little infectivity but did demonstrate the ability to interfere with infectious virus replication in interference assays. This was demonstrated by treating control STE-137 cells with virus of 1.29 g/cc density for 12-24 h, a period
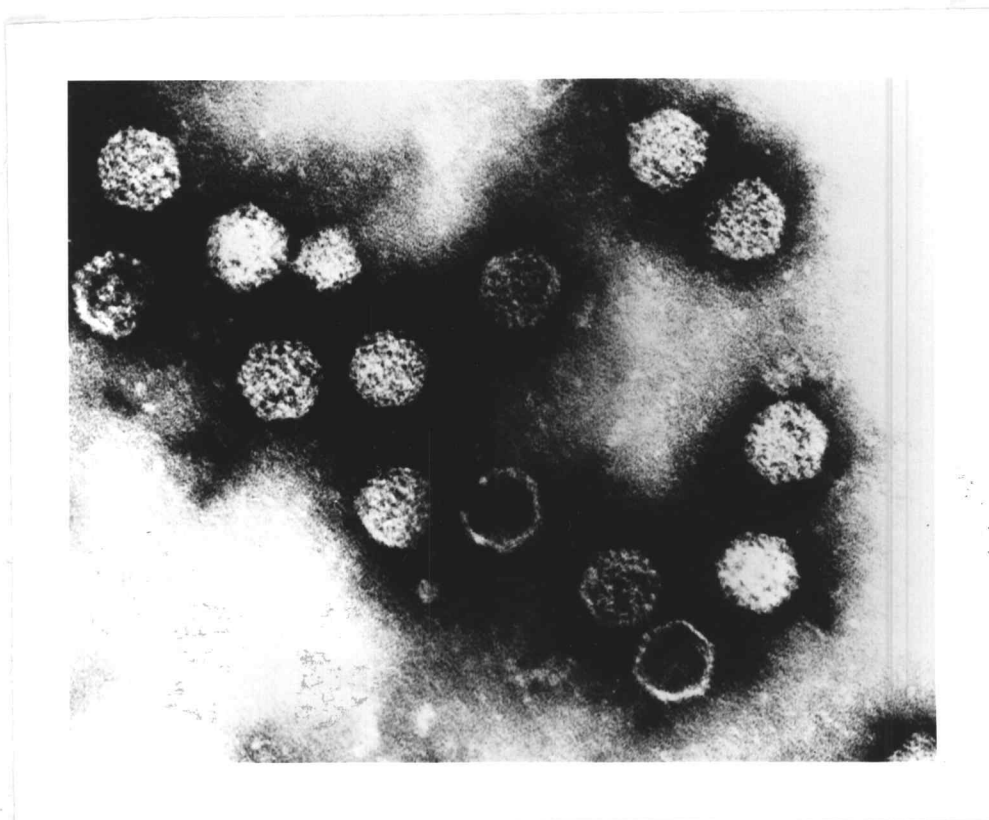
Figure 5. Electron micrograph of infectious pancreatic necrosis
virus (CL-IPNV) negatively stained with 1% phosphotungstic
acid. The mean diameter of the virions in this hydrated
state is 70 nm.
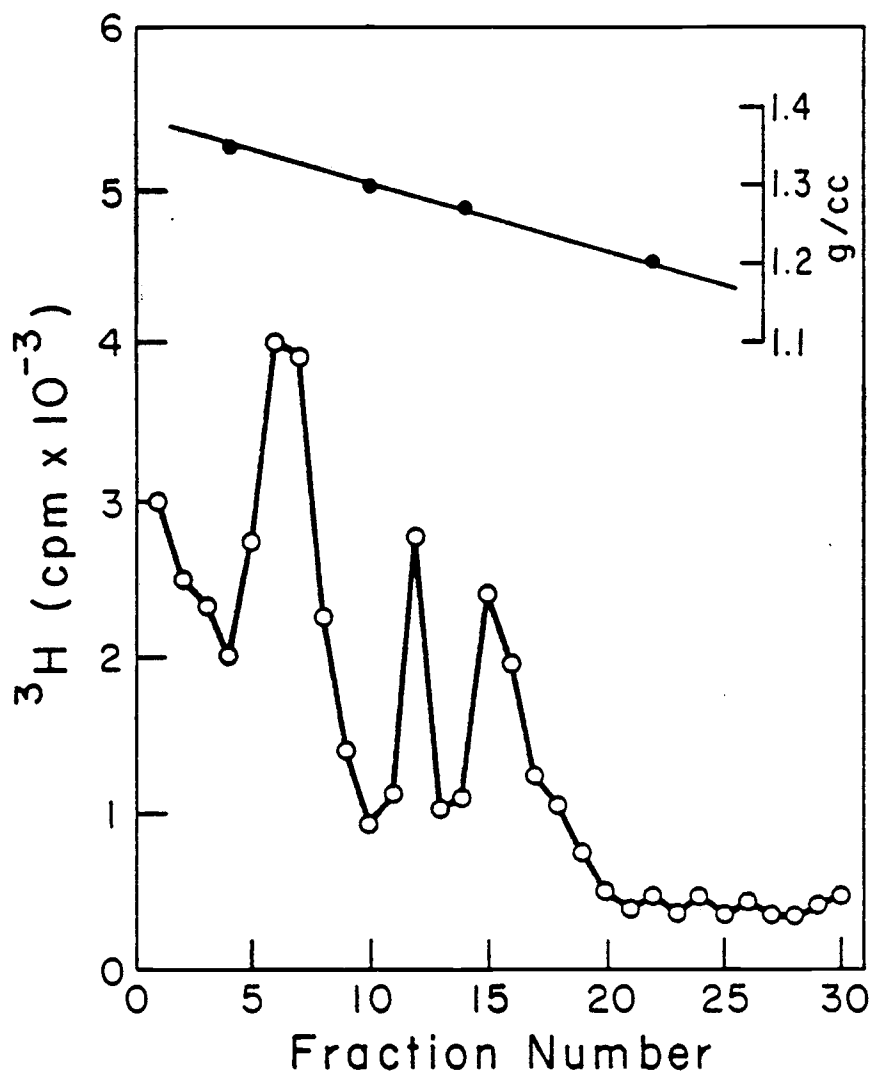
Figure 6. Isopycnic centrifugation in cesium chloride of infectious pancreatic necrosis virus (CL-IPNV) from lytically infected STE-137 cell cultures. Cells were infected at an MOI of 0.01 and the viral RNA labeled with (5-^{3}H) – uridine.
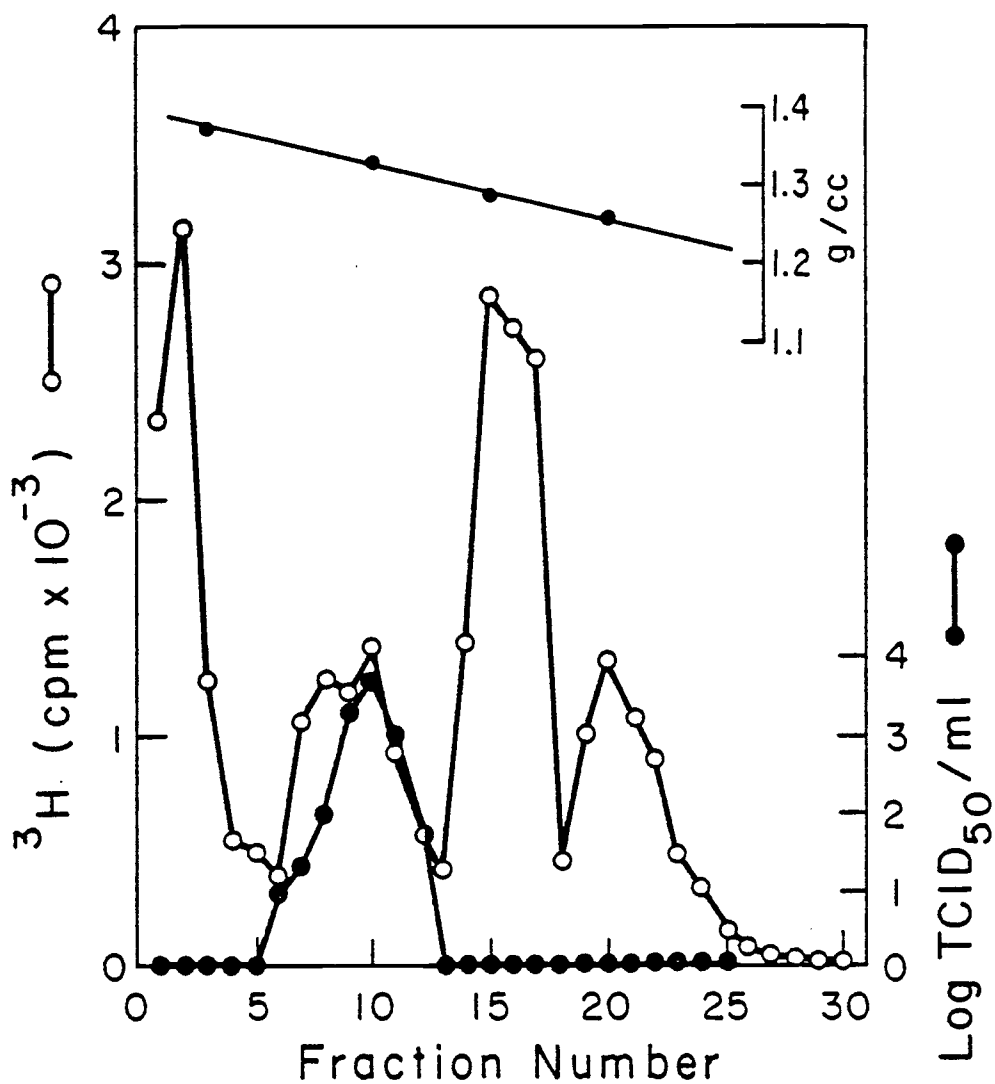
Figure 7. Isopycnic centrifugation in cesium chloride of infectious pancreatic necrosis virus (CL-IPNV) from persistently infected STE-137 cell cultures. The viral RNA was labeled with $(5-^3H)$ - uridine (O) and the infectivity (●) determined by $TCID_{50}$ analysis.

determined by Nicholson and Dunn (1974) to be optimal for detecting

the activity of defective interfering virus, followed by a challenge

with infectious virus. Cells treated in this manner yielded 300 fold

less infectious virus than untreated controls challenged with the same

amount of CL-IPNV.

Virus banding at a density of 1.29 g/cc was unstable to repeated

cycles of dialysis and ultracentrifugation and could not be observed

by electron microscopy. The numbers of these virus were probably

below the levels detectable by this technique.

The density of defective virus from STE-137 PI cell cultures was

less than infectious virus (1.29 compared to 1.33 g/cc) indicating that

there was probably less viral RNA in these particles. The production of

these particles during viral persistence has two important consequences.

Defective virus can reduce not only the (1) amount of virus produced

by a given cell population but also (2) the CPE associated with infectious

virus release.

Autointerference

If defective interfering virus are produced and released by

STE-137 cells persistently infected with CL-IPNV, their activity

(interference with infectious virus replication) should be detectable

in the culture fluids. During the titration of virus released from

the PI STE-137 cell line by $TCID_{50}$ assay, it was observed that the

undiluted or lower dilutions of virus often did not exhibit any or

minimal CPE. Similar autointerference had been reported with IPNV

(Malsberger and Cerini, 1965 and Nicholson and Dunn, 1974) as a

result of the production of defective interfering virus by repeated

undiluted passages of virus stocks. The yields of infectious virus

from cells demonstrating autointerference are characteristically lower

than yields from cells lytically infected with higher dilutions of the

same virus inocula. The yields of infectious virus from cells

inoculated with undiluted culture fluids containing virus from PI

STE-137 cells at passage number 29 and 34 were as great as 1,585 fold

less than diluted virus preparations (Table 8). These results further

supported the production and release of defective interfering virus by

PI STE-137 cells.

Characteristics of Carrier Brook Trout

Release of Infectious Virus in Feces

The persistence of IPNV in carrier brook trout was monitored to

determine if the levels and patterns of virus release were similar to

those observed in PI cell lines. The concentrations of IPNV released in

the feces of 14 carrier brook trout sampled weekly for a period of 10

weeks is depicted in Table 9. Virus levels often fluctuated over a 100

to 1000 $TCID_{50}$ range between weekly samples of the feces from the same

carrier fish. A majority of the carriers continually shed low levels of

virus with intermittent episodes of increased virus production (Figure

8). All 14 carriers released infectious virus in the feces

during at least one of the weekly sampling periods. Nine of the

carriers were continuing to release infectious virus in the feces at

Table 8. Yields of infectious virus from cells inoculated with undiluted and diluted culture fluids of STE-137 cells persistently infected with infectious pancreatic necrosis virus.

Source of Culture Fluid	Passage No.	Dilution[a]	CPE	Yield	Ratio diluted/ undiluted
STE-137/CL-IPNV	P 29	10^0	–	$10^{5.5}$[b]	
		10^{-2}	+	$10^{8.7}$	1585
STE-137/CL-IPNV	P 34	10^0	–	$10^{6.0}$	
		10^{-1}	–	$10^{5.5}$	0.32
		10^{-2}	+	$10^{8.0}$	100
		10^{-5}	+	$10^{8.7}$	501

[a]Cell culture fluids from STE-137/CL-IPNV cultures were applied directly or following a 10-fold dilution in MEM-10 to cells in 96 well dishes.

[b]Expressed as $\log_{10}$ $TCID_{50}$/ml.

Table 9. Concentrations of infectious pancreatic necrosis virus detected in the feces of individual carrier brook trout sampled weekly over a ten-week period.

Fish No.	Week No.									
	1	2	3	4	5	6	7	8	9	10
1	3.1[a]	1.9	2.5	<1.6	<1.6	<1.6	2.5	3.0	<1.6	<1.6
2	3.4	3.0	3.1	<1.6	<1.6	<1.6	3.3	3.5	2.6	<1.6
3	<1.6	<1.6	<1.6	2.0	3.3	2.5	<1.6	3.0	3.5	3.6
4	<1.6	3.0	<1.6	2.8	<1.6	3.2	3.0	3.5	3.5	3.5
5	3.1	<1.6	2.6	3.1	2.6	3.5	3.5	3.5	3.2	3.2
6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	2.0
7	2.8	2.9	2.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
8	<1.6	<1.6	2.0	2.8	3.0	2.6	2.8	Died	--	--
9	<1.6	<1.6	1.8	<1.6	2.8	2.5	<1.6	<1.6	<1.6	2.8
10	2.6	3.1	2.0	3.4	<1.6	2.5	2.6	<1.6	<1.6	2.6
11	<1.6	2.8	<1.6	3.0	<1.6	<1.6	<1.6	<1.6	<1.6	1.8
12	<1.6	2.9	<1.6	<1.6	<1.6	<1.6	<1.6	3.5	3.0	<1.6
13	3.1	2.8	2.6	5.0	4.5	4.0	3.8	3.7	3.0	2.4
14	3.0	3.0	4.5	<1.6	2.0	2.8	3.5	3.5	2.6	3.2

[a]Exponent of $\log_{10}$ of the $TCID_{50}$ per gram.
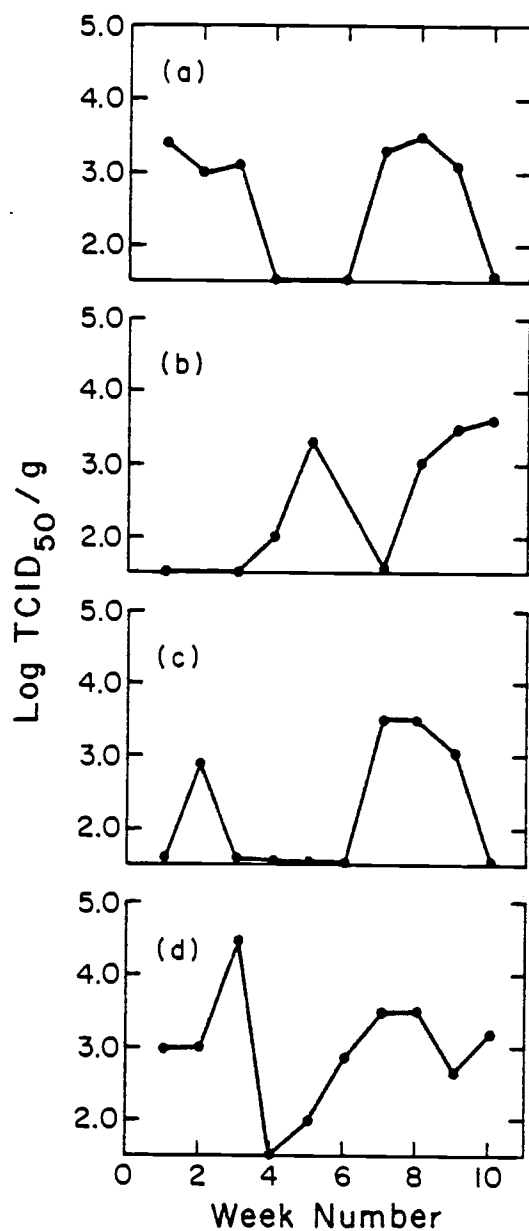
Figure 8. The concentrations of infectious pancreatic necrosis virus (CL-IPNV) detected in the feces of 4 individual carrier brook trout sampled over a 10 week period (a) fish no. 2 (b) fish no. 3 (c) fish no. 12 (d) fish no. 14. Fish numbers are shown in Table 5.

the 10th weekly sample. Interestingly, fish number 6 which had not been releasing detectable levels of virus from week 1 through 9, was shedding virus for the first time at week 10.

Virus Levels in Tissues

At the end of the 10 week fecal sampling period the carrier trout were sacrificed. Certain tissues were examined to determine the concentrations of virus present. The concentrations of IPNV recovered from carrier trout tissues at the time of sacrifice are displayed in Table 10. Tissue from the pancreas-caeca contained the highest concentrations of IPNV. The kidney tissues contained lower and the spleen the least concentrations of CL-IPNV compared to the pancreas-caeca. The feces, also good indicators of virus infection, were 10 to 100 fold less than the levels found in the pancreas-caeca.

Neutralizing Antibody Titers

Serum neutralization tests for anti-CL-IPNV antibody were performed to determine if the levels of antibody could be correlated to virus concentrations in the tissues tested. Anti-IPNV neutralizing antibody was detected in all carrier brook trout sera tested. The levels of antibody found in the sera of 7 carrier brook trout was highly variable (Table 10). Titers ranged from 1:25 to greater than 1:4800 for the dilution of serum at which 50% of test cultures containing CL-IPNV were neutralized. In addition the titers of neutralizing anti-virus antibody could not be correlated with the concentrations of virus detected in any of the tissues. Sera from 5 uninfected brook

Table 10. Concentrations of infectious pancreatic necrosis virus detected in the tissues and the anti-virus neutralizing titers of serum from individual carrier brook trout.

Fish No.	$TCID_{50}/g$ [a]				Titer [b]
	Spleen	Kidney	Pancreas-Caeca	Feces	Serum
2	<1.6	<1.6	<1.6	<1.6	25
3	<1.6	3.7	5.3	4.0	3200
4	<1.6	<1.6	3.5	5.0	50
5	<1.6	5.5	5.5	3.5	324
12	2.5	2.7	5.5	3.0	648
13	<1.6	2.5	5.5	3.0	4800
14	<1.6	5.0	5.5	3.5	2236

[a] Expressed as the exponent of log_{10} of the $TCID_{50}$ per gram.

[b] Expressed as the reciprocal of the dilution giving a 50% endpoint.

trout from the same stock of fish used to establish carriers were held

as controls, had no detectable neutralizing antibodies at dilutions of

1:25.

Electron Microscopy

Thin sections prepared from the caeca and kidneys of seven carrier

trout were examined by electron microscopy to determine if structures

resembling the defective virus in PI cell lines could be seen.

Electron microscopic examinations failed to demonstrate the presence of

IPNV virions. Particles of approximately the correct size and shape

were observed but were never in sufficient numbers to determine they

were virions. The inability to locate virions by electron microscopy

may have reflected the low incidence of infected cells in the carrier

trout tissues. Finding one infected cell in a hundred or thousand by

examining thin sections of just a few cells may explain the difficulty

in detecting these virions.

Infectious Center Assays

The number of cells in carrier trout tissues that were producing

infectious virus was determined by infectious center assays. The

percentage of the cells acting as infectious centers ranged from 0.06

to 0.5% for the five carrier brook trout kidneys examined (Table 11).

As the number of infectious centers increased, so did the concentration

of virus recovered from the kidney of that carrier trout. Infectious

center assays determined that only a small percentage of the total

Table 11. Percent infected cells in kidney tissues
of infectious pancreatic necrosis virus
carrier brook trout as determined by
infectious center assays.

Fish No.	Viable Cell Counts	Infectious Centers[a]
2	4.2×10^5	0.0
3	2.5×10^5	0.4
12	3.3×10^5	0.06
13	1.5×10^5	0.2
14	2.7×10^5	0.5

[a]Expressed as the percent of total cells acting as
infectious centers.

cells comprising the kidney tissue are actively producing infectious

IPNV. The low incidence of virus producing cells explains the

difficulty in their detection by electron microscopic examinations.

DISCUSSION

Infectious pancreatic necrosis virus established persistent

infections both in live brook trout and two salmonid cell lines

derived from steelhead trout and chinook salmon embryonic tissues. As

the study progressed it became evident that persistently infected cell

lines shared many characteristics with IPNV infected carrier brook

trout and suggested that these virus-host cell interactions might be

identical in vitro and in vivo. The mechanisms involved in the

establishment and maintenance of viral persistence in these cell lines

were investigated to determine if they might serve as in vitro models

of IPNV persistence in carrier brook trout.

Persistent viral infections in cell cultures have been established

with most of the taxonomic groupings of RNA viruses (Rima and Martin,

1976). Viral persistence initiated in many of these cultures has been

traced to the presence of defective interfering (DI) or temperature

sensitive (ts) viruses, or both, in the original virus inoculum

(Joklik, 1977). Although the virus used in this study (CL-IPNV) did

not demonstrate temperature sensitivity, it did produce DI virus.

Nicholson and Dunn (1974) and MacDonald and Yamamoto (1978) showed

serial undiluted passages of IPNV in RTG-2 and CHSE-214 cells generated

DI virus that inhibited infectious virus replication and promoted

cell survival. Defective interfering CL-IPNV were prepared in CHSE-214

cells by serial undiluted passages to determine if they would increase

the frequency of persistent infections. Virus inocula prepared by

this procedure routinely established persistent infections in CHSE-214 cells. In contrast, virus inocula repeatedly plaque purified to reduce the levels of DI viruses infrequently initiated persistent infections. This indicates that high concentrations of DI virus often results in persistent infection while low concentrations allow complete or infectious virus to cause complete cell destruction. The establishment of persistent infections in STE-137 cells was not as clearly related to the effect of DI CL-IPNV. Challenge of STE-137 cells with CL-IPNV containing low levels of DI viruses was characterized by extensive CPE with few surviving cells. Subsequent culture of these surviving cells indicated that persistent infection may occassionally result from virus inocula that contain low levels of DI IPNV.

Once established, the characteristics of these two cell lines persistently infected with CL-IPNV were examined. Certain features of CL-IPNV carrier brook trout were also examined in this study and when possible were compared to those of PI cell lines. Infectious virus released by both PI cell lines was detected in the culture fluids at each weekly subculturing (Figure 1). Similar levels of CL-IPNV were also recovered in the feces of carrier brook trout. These results are in agreement with those previously reported by Yamamoto (1975a) and Billi and Wolf (1969). Billi and Wolf (1969) compared the levels of IPNV in the feces of carrier brook trout sampled periodically over 9 months. They concluded that fish shedding high levels of IPNV tended to maintain that level and fish releasing lower levels maintained that output. In contrast, fecal samples taken at regular (weekly) intervals from

CL-IPNV carrier brook trout showed that infectious virus release was more dynamic and often involved significant fluctuations in virus concentrations from one week to the next. The levels of infectious virus released into the culture fluids of PI cell lines also fluctuated with each weekly sampling indicating that this pattern of virus shedding is characteristic of CL-IPNV persistence both in vitro and in vivo.

The quantities of virus released from both PI cell lines were substantially less (10^3 to 10^7 fold) than yields of IPNV from lytically infected cells. These low yields could result from restricting the number of cells in the population participating in viral replication. The number of cells producing infectious virus in both PI cell lines was determined by infectious center assays to be 0.1 to 1.0% of the total cell population. Comparable percentages (0.06 to 0.5) of cells acting as infectious centers were found in kidney tissues from CL-IPNV infected carrier brook trout. Reno (1976) obtained similar results using immunoperoxidase staining to determine that only a few cells in the tissues from carrier trout were infected. The production of infectious virus by only a few cells during viral persistence was further supported by immunofluorescent examinations of PI cell lines. In both PI STE-137 and CHSE-214 cell lines, 1% or less of the cells contained detectable amounts of viral antigens. Most of these cells exhibited an intensely staining distorted cytoplasm which indicated that the production of virus probably lead to their destruction. The remainder of the cells in these PI cell lines did not contain

detectable viral antigens or act as infectious centers. These cells
however were solidly protected from the effects of infectious virus as
demonstrated by the inability of high MOI challenges with CL-IPNV to
induce CPE or increase the number of cells staining positively by
immunofluorescent techniques. The protection of these cells is quickly
disrupted by the passage of PI cell lines in growth medium containing
anti-IPNV rabbit serum. Three passages in medium containing antibody
followed by one passage without antibody renders most of the cells in
the PI cultures susceptible to infectious virus-induced CPE. A
similar effect results from the addition of an excess of uninfected
cells to a few cells from PI cell lines. The passages of PI cells in
growth medium containing anti-virus antibody probably allows the growth
and division of uninfected cells by blocking the diffusion of virus
from virus producing cells. Persistently infected STE-137 cells that
were passaged 7 times in growth medium containing antibody and 2 times
in medium without antibody no longer produced infectious virus.
Teninges et al. (1979) has reported drosophila cells persistently
infected with drosophila X virus (IPNV-like) were also cured by
repeated passages of these PI cells in growth medium containing anti-
virus antibody.

A second mechanism that would result in the low yields of virus
released from PI cell lines is the modulation or control of the amount
of infectious virus produced by each cell. Interferon, temperature
sensitive and DI viruses functioning alone or cooperatively have the
ability to regulate the yields of infectious virus produced per cell

(Joklik, 1977). Persistently infected cell cultures that regulate infectious virus replication by the production of interferon are characterized by their resistance to superinfection with taxonomically diverse viruses (Sekellick and Marcus, 1978). Both cell lines (PI with CL-IPNV) in this study were susceptable to challenges with three taxonomically unrelated viruses from fish, indicating that interferon was probably not being produced. The absence of interferon in PI CHSE-214 may result from a defect in this cell lines ability to synthesize this protein. MacDonald and Kennedy (1979) have shown that CHSE-214 cells did not produce interferon in response to lytic infections with IPNV or synthetic polymers, while two other fish cell lines from rainbow trout (RTG-2) and fat head minnow (FHM) did.

Interferon has been isolated and partially characterized from RTG-2 cells (deSena and Rio, 1975) and live rainbow trout (deKinkelin and Dorson, 1974) in response to IPNV infection. These interferons exhibited a broad anti-virus activity and cell specificity, characteristic of mouse and human interferons (Marx, 1979). Using the same techniques as deSena and Rio (1975) and deKinkelin and Dorson (1974), anti-viral activity indicative of interferon could not be detected in the culture fluids of either STE-137 or CHSE-214 cell lines persistently infected with CL-IPNV. The plaquing efficiency and yields of IHNV from PI compared to normal cell lines clearly indicated that heterologous virus replication is not hindered as a result of persistent infection with IPNV. These results suggest that neither PI cell line relies on the production of interferon to control infectious virus replication.

Attempts to detect interferon in carrier brook trout sera were
hampered by the absence of a cell line from the genus Salvelinus.
Repeated efforts to establish a cell line from brook trout tissues
failed. Sera drawn from carrier brook trout sacrificed following a 10
week fecal sampling period were tested on RTG-2 cells to determine if
interferon present might induce activity in cells of a different genus.
Cells treated in this manner and later challenged with Metolius River
strain of IHNV did not exhibit plaque reduction, an activity that would
indicate the presence of interferon. These results although suggestive
of the absence of interferon in carrier brook trout sera do not preclude
its presence. Final testing will require the establishment of a cell
line from the genus Salvelinus, something no one has yet been able to do.

Temperature sensitive (ts) viral mutants are also known to control
infectious virus production in PI cell lines and persistently infected
animals (Graham, 1977 and Rima and Martin, 1976). Youngner and
Quagliana (1976) found that ts mutants recovered from culture fluids of
L cells persistently infected with VSV tended to be noncytocidal and
interfered strongly with the replication of wild type virus. Youngner
et al. (1976) observed a rapid selection for these ts mutants among
the viruses released from L cells persistently infected with wild type
VSV. Six cell passages following the establishment of persistent
infection, virus titrations of culture fluids at 39.5 compared to 32°C
($^{39.5}$/32°C) were 2.1 x 10^{-4} or less, indicating that ts VSV was the
major virus species released.

Both cell lines persistently infected with CL-IPNV in this study were propagated at 16°C which is below the optimal temperature of 20-22°C (Malsberger and Cerini, 1965) for IPNV replication. The culture fluids from both PI STE-137 cells at passage 41 and CHSE-214 at passage 21 were titrated at 16 and 22°C to determine if the virus released demonstrated temperature sensitivity. The virus released by both PI cell lines replicated slightly more efficiently at 16 than 22°C. This difference was substantially less than those observed with L cells persistently infected with VSV in which ts virus were known to be important in controlling infectious viral replication. By passage 8, L cells persistently infected with VSV released only ts VSV which did not replicate at elevated temperatures. In contrast, both cell lines persistently infected with CL-IPNV continued to release substantial levels of virus into the culture fluids at passages 21 (CHSE-214) and 41 (STE-137) that replicated at elevated temperatures (22°C). In addition, both PI cell lines were shifted to 22°C to determine if elevating the temperature to that optimal for IPNV replication might induce CPE and infectious virus replication. Increasing the temperature resulted in slight declines in infectious virus production accompanied by enhanced cell growth with both PI cell lines. These results indicated the temperature of incubation of PI cell lines and the temperature sensitive virus released are probably not as important as other factors in controlling the levels of infectious virus released during IPNV persistence.

The amount of infectious virus produced by an infected cell may also be limited by the presence or production of defective interfering (DI) viruses. Defective interfering viruses are identified by their ability to specifically suppress the replication of homologous (taxonomically related) but not heterologous viruses (Joklik, 1977). These viruses are often associated with chronic or persistent infections in which the yields of infectious virus are lower than acute or lytic infection (Holland and Villarreal, 1975b). It was therefore necessary to determine in this study, if DI viruses might also be produced by cell lines persistently infected with CL-IPNV. An electron microscopic examination of PI STE-137 cells at passage 6 revealed the presence of unique ultrastructural features in the cytoplasm of certain cells. Particles of approximately the same size (55-57 nm) and shape of IPNV were detected within cytoplasmic vacuoles of PI cells but were not present in lytically infected or uninfected control cells. Ahne (1977) observed similar structures in CHSE-214 cells persistently infected with IPNV and concluded that they were probably DI viruses. The particles observed by Ahne (1977) and those demonstrated in this study were considered presumptive evidence that DI viruses were produced during persistent IPNV infections. Labeled virus (^{3}H) extracted from PI STE-137 cells were isopycnically centrifuged in CsCl. The bouyant densities and infectivities of particles were determined by measuring the amount of radioactivity following fractionation of the CsCl gradient. Most particles extracted from PI STE-137 cells formed a band at a density of 1.29 g/cc with a smaller amount at 1.33 g/cc.

Infectivity experiments determined that infectious virus banded at a density of 1.33 g/cc. Viruses extracted from lytically infected cells formed a band at 1.33 g/cc with only a small band at 1.29 g/cc. Uninfected control cells processed in the same manner showed no virus bands at either density. Clearly, virus production in PI cells differed from that in lytically infected cells by an increased production of a virus species (1.29 g/cc) that showed little or no infectivity.

The effect of this virus species on the replication of infectious virus (1.33 g/cc or stock CL-IPNV) was tested by treating control STE-137 cells with material of 1.29 g/cc density followed by a challenge with infectious virus. Cells treated in this manner demonstrated reduced CPE and infectious virus yields compared to cells treated with only infectious virus. This indicated that bands containing virus at 1.29 g/cc isolated from PI STE-137 cells exhibited two important characteristics attributed to DI viruses: (1) the ability to interfere with infectious virus replication and (2) lighter bouyant densities than infectious viruses. MacDonald and Yamamoto (1978) found that DI VR-299 viruses produced by lytically infected CHSE-214 cells interfered strongly with infectious virus replication. Defective interfering viruses in that study had densities that ranged from 1.28 to 1.33 g/cc. The wide range of DI particle densities observed by MacDonald and Yamamoto (1978) may be a property of that virus strain (VR-299) or cell line. The species of DI VSV particles produced by VSV is known to be a function of the virus strain and cell line, or both (Huang,

1977). The virus employed in this study (CL-IPNV) seems to produce only one species of DI particles in STE-137 cells and these particles have a bouyant density of 1.29 g/cc compared to 1.33 g/cc for infectious virus.

The production of DI CL-IPNV during viral persistence explains several of the features observed with PI cell lines and carrier brook trout. The levels and patterns of infectious virus released both *in vivo* and *in vitro* are characteristic of persistent infections involving DI viruses (Rima and Martin, 1976). The fluctuating release of infectious virus may result from alternate cycles of production of DI and infectious CL-IPNV, a situation observed with hamster cells (CHO) persistently infected with VSV (Palma and Huang, 1974). When infectious virus concentrations are high, DI concentrations would be low, conversely when infectious virus concentrations were low, DI concentrations would be high. Titrations performed on PI cell culture fluids or carrier trout tissues during periods of high DI concentrations should exhibit the same autointerference phenomenon that is observed when high DI concentrations are purposely generated in cell cultures by serial undiluted passages of IPNV stocks. Auto-interference was observed with virus in the culture fluids from PI STE-137 cells at passage 29 and 34 (Table 6). The reductions in virus yields and CPE that characterized this viral interference were remarkably similar to that observed in RTG-2 and CHSE-214 cells with IPNV stocks containing high concentrations of DI viruses (Nicholson and Dunn, 1974 and MacDonald and Yamamoto, 1978). The viral interference reported by Nicholson and

Dexter (1975) with IPNV from carrier brook trout tissues was also characterized by similar reductions in virus yields and CPE. Viral interference was not observed during titrations of carrier brook trout tissues in this study. Fecal material, pancreas-caeca and kidney tissues all demonstrated degrees of toxicity to CHSE-214 cells at dilutions that would have permitted the detection of autointerference observed with the culture fluids from PI cell lines.

The production of DI viruses by cell lines persistently infected with CL-IPNV also explains their response to superinfection with homologous and heterologous viruses. Both PI cell lines were resistant to superinfection with several serologically diverse strains of IPNV but susceptable to 3 heterologous viruses. These results are consistent with the specific nature of viral interference caused by DI viruses but in marked contrast to the nonspecific interference that results from interferon production (Sekellick and Marcus, 1978).

In addition, DI viruses may indirectly control the number of cells participating in infectious virus replication. A majority of the cells (99% or more) in both PI cell lines were protected from superinfection with homologous virus. These cells do not exhibit positive immunofluorescence which indicates that virus or viral products are not being produced. This suggests that DI viruses must diffuse from virus producing cells (presumably producing infectious virus also) to the surrounding cells. If DI IPNV shows a one-hit to protect kinetics as MacDonald and Yamamoto (1978) has proposed, cells infected with as few as one DI would be protected from infectious virus. Cells protected

by DI infection must block infectious virus replication before the

production of viral polypeptides. If viral replication was not blocked

at an early stage, protected cells would contain viral antigens

detectable by immunofluorescent techniques, as found in cell lines

persistently infected with measles, togavirus and arenavirus

(Rima and Martin, 1976).

The protection afforded to most of the cells in lines

persistently infected with CL-IPNV must begin early, perhaps with

the first cell producing sufficient DI viruses to protect neighboring

cells. This is supported by immunofluorescent examinations of PI cell

lines prior to their first subculturing. In these cultures the number

of cells which stained positively for viral antigens has already been

established at 1% or less of the cell population. These results suggest

that DI CL-IPNV may function in persistence by reducing the number of

cells that are involved in viral replication by protecting most of the

population from subsequent infection and replication of infectious virus.

Virus persistence is probably maintained by a small percentage of cells

that are not protected prior to contact with infectious virus. It is

also conceivable that the protection confered by DI viruses is not always

completely effective. In such cases the occasional loss of protection

could give rise to a small percentage of virus producing cells.

The persistent nature of IPNV infections has long been recognized

in carrier trout (Wolf, 1966) and recently in cell cultures (Ahne, 1977

and Hedrick et al., 1978b). Viral persistence is characterized by the

fluctuating release of low levels of infectious virus in the absence

of external signs (in vivo) or cytopathic effects (in vitro). This results from restricting the number of cells participating in viral replication in both PI cell lines and carrier brook trout tissues. It was further determined in PI cell lines that infectious virus yields per cell can be controlled by the production of DI viruses.

Viral persistence in carrier brook trout has additional complexities that are not encountered in these PI cell lines. The live trout has the ability to provide an immune response to viral infection. Circulating anti-virus antibodies were detected in the sera of all carrier brook trout examined in this study. This response however was highly variable between individual fish. Titers of anti-CL-IPNV neutralizing antibodies ranged from 25 to 4800 ND_{50}/ml and could not be correlated with levels of infectious virus detected in the feces or tissues. Similar results were reported by Yamamoto (1975b) and Reno et al. (1978) following examinations of IPNV carrier brook and rainbow trout sera. The antibody responses of trout that are carriers of bacterial diseases (i.e. kidney disease, furunculosis, columnaris) have also been shown to be highly variable and not correlated to resistance to the disease agent (Fryer et al., 1976). This may result from the sequestering of the pathogens at sites unavailable to circulating antibody or to possible defects in the immune response (Wolf et al., 1969 and Reno et al., 1978). The ability to cure persistent infections in PI cell cultures by repeated passages in growth medium containing anti-virus antibody suggests if antibody can make effective contact with virus producing cells, infection will be terminated.

Regardless of the complexity of viral persistence in carrier trout the mechanisms which function at the virus host cell level seem analogous to those in PI cell lines. The similarities between infections in carrier brook trout and PI cell lines suggest that similar mechanisms are involved in viral persistence. Persistently infected cell lines can therefore serve as invaluable models in understanding the biological as well as biochemical interactions between virus and host cells which result in persistent viral pathogenesis. The use of these cell lines as models might be extended to examine the cell mediated immunity of carrier trout. The interactions of lymphocytes isolated from carrier brook trout with virus producing cells _in vitro_ in the absence or presence of antibody may give insights into the role of cell mediated immunity in viral persistence.

The establishment of persistent infections with IPNV in cell lines from salmonids and nonsalmonid fish that are not susceptible to virus induced mortality might be examined and compared to the persistence in cells of susceptable salmonids. The characteristics of viral persistence in various cell lines may help to explain the variable response to virus infections by these different host species _in vivo_. It might then be possible to examine the genetics of the host cell that determine whether infection is acute, abortive or persistent.

SUMMARY AND CONCLUSIONS

1. Persistent infections with CL-IPNV were established in both STE-137
    and CHSE-214 cell lines.

2. Virus inocula containing high concentrations of defective
    interfering CL-IPNV routinely establish persistent infections in
    CHSE-214 cells. Persistent infections were established but
    less frequently in CHSE-214 and STE-317 cells with virus inocula
    containing few defective interfering CL-IPNV.

3. Both persistently infected cell lines released low levels of
    CL-IPNV that were detected in the supernatant fluids at each weekly
    subculturing. The levels and patterns of virus release were
    similar to those observed in CL-IPNV infected carrier brook trout
    feces.

4. The low levels of infectious virus released during IPNV persistence
    is accomplished by restricting the number of cells (0.06 to 1.0%)
    producing infectious virus in both persistently infected cell lines
    and carrier brook trout tissues.

5. Immunofluorescent examinations of persistently infected cell lines
    indicated that 1% or less of the cells produce detectable amounts
    of CL-IPNV antigens. These cells were often in advanced stages of
    infection suggesting that death resulted from viral replication.

6. The majority of the cell population in persistently infected cell lines is protected from superinfection with homologous but not heterologous viruses.

7. Propagation of persistently infected cell lines in growth medium containing anti-virus antibodies disrupts the protection of a majority of the cells, presumably by preventing the diffusion of viral products (defective interfering viruses) from virus producing cells.

8. Prolonged propagation of persistently infected cell lines in growth medium containing antibody cures persistent infection probably by the selective removal of the virus producing cells at each subculture.

9. Interferon was not detected in either persistently infected cell line. Although interferon was not detected in carrier brook trout sera tested on RTG-2 cells, this does not preclude its presence.

10. Although the virus released from persistently infected cell lines exhibited slight temperature sensitivity, shifting cultures to temperatures favorable to wild type IPNV replication had no effect on viral persistence and indicated that temperature and temperature sensitive virus mutants are probably not involved in viral persistence in these cell lines.

11. Defective interfering viruses produced by STE-137 cells
    persistently infected with CL-IPNV were isolated by isopycnic
    centrifugation in CsCl and had a bouyant density of 1.29 g/cc.
    Infectious virus also produced by these cells had a density of
    1.33 g/cc.

12. Defective interfering virus isolated from persistently infected
    STE-137 cells suppressed the replication of infectious virus
    produced by control STE-137 cells pretreated with defective
    interfering virus and then challenged with CL-IPNV. Defective
    interfering virus therefore have the ability to reduce the
    amount of infectious virus produced by infected cells.

13. Resistance to homologous virus superinfection, fluctuating releases
    of infectious virus, autointerference and low virus yields may
    all be manifestations of defective interfering virus production by
    cells persistently infected with CL-IPNV.

14. The patterns and levels of infectious virus released from carrier
    brook trout suggest that defective interfering IPNV is also produced
    in vivo.

15. Persistently infected cell lines serve as invaluable models of the
    biological and biochemical interactions between virus and host
    cells that result in viral persistence.

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