A study of innate antiviral mechanisms using fish cell lines

by

Stephanie Johanna DeWitte-Orr

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I hereby declare that I am sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Understanding basic antiviral mechanisms in vertebrates is essential for developing methods to enhance antiviral responses and promote human and animal health. In fish these antiviral mechanisms are poorly understood, but are important to understand because of the devastating impact of viral diseases on aquaculture. Therefore, the antiviral responses of a rainbow trout macrophage-like cell line, RTS11, and two non-immune cell lines, the rainbow trout fibroblast RTG-2 and Chinook salmon embryo CHSE-214 were studied. Three antiviral responses were first characterized using the viral mimic, synthetic double-stranded RNA (poly IC), and then their induction was investigated using Chum salmon reovirus (CSV). The responses were: 1) apoptosis, which is programmed cell death and a primitive antiviral defense; 2) homotypic aggregation (HA), which is clustering of like immune cells; and 3) expression of Mxs, which are antiviral proteins belonging to GTPase super-family. Some of these antiviral mechanisms were investigated using a novel continuous cell line, PBLE, developed from a peripheral blood leukocyte preparation of the American eel, *Anguilla rostrata*.

RTS11 was exceptionally susceptible to apoptosis. The cells died at lower concentrations of poly IC and other agents, including the translation inhibitor, cycloheximide (CHX), and fungal metabolite, gliotoxin. Death was predominantly by apoptosis, as judged by DNA ladders, nuclear fragmentation, and protection by caspase inhibitors. By contrast, the other two cell lines died most commonly by necrosis, when death did occur. Co-treating RTS11 with CHX greatly sensitized the cells to poly IC. Based on the protection afforded by inhibitors of dsRNA-dependent protein kinase (PKR), RTS11 apoptosis induced by poly IC with CHX co-treatment but not gliotoxin was mediated by PKR. As macrophages are likely among the first cells to contact viruses during an infection *in vivo* and are mobile, the sensitivity of RTS11 to dsRNA killing could reflect a protective mechanism by which virus spread is limited by the early death of these first responders.

HA of RTS11 was induced by poly IC. HA required divalent cations and was blocked by CHX and by PKR inhibitors. This suggested that HA induction was PKR-mediated and involved the synthesis of new cell surface molecule(s), possibly galectins. As an antiviral mechanism, HA induction by dsRNA could be interpreted as an initial protective response, allowing cell localization at the site of infection, but once translation becomes inhibited, apoptosis ensues.

Mx was induced by poly IC in RTS11 and RTG-2 as judged by RT-PCR. Western blotting revealed constitutive Mx expression more consistantly in RTS11, but induction by poly IC in both cell lines. Medium conditioned by cells previously exposed to poly IC and assumed to contain interferon also induced *Mx* transcripts in RTS11 but not RTG-2. In RTS11, poly IC activated PKR

activity, and PKR inhibitors blocked *Mx* induction, which is the first demonstration of PKR mediating Mx expression.

The dsRNA virus, CSV, also induced apoptosis, HA, and Mx expression, but in some cases contrasting with poly IC experiments. CSV induced apoptosis in RTG-2 and CHSE-214 but not in RTS11, and HA induction by CSV in RTS11 was not dependent on PKR. Mx induction was sustained in RTG-2 and transitory in RTS11; however, both cell lines supported CSV replication.

The novel cell line, PBLE, was also characterized in this study. PBLE was derived from an adherent culture of peripheral blood leukocytes from the American eel, *Anguilla rostrata*. PBLE were found to grow over a wide range of temperatures and fetal bovine serum (FBS) concentrations. This cell line was able to undergo apoptosis in response to gliotoxin. PBLE was also susceptible to a number of viruses, including CSV; however, CSV infection did not lead to apoptosis.

This study suggests that antiviral responses are likely numerous and overlapping and depend on cell type and virus. Understanding them should lead to novel methods for protecting fish from viral diseases. More specifically, using cell lines such as PBLE may aid in the understanding of species specific and perhaps even cell type specific antiviral mechanisms.

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DEDICATION

This thesis is dedicated to those I love. Thank you, for everything you do.

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LIST OF ABBREVIATIONS

aa	amino acid
AB	alamar Blue
AMD	actinomycin D
2-AP	2-aminopurine
Apaf-1	apoptosis-activating factor-1
AqRV	aquareovirus
ARV	avian orthoreovirus
Bap	benzo[a]-pyrene
BLCAM	B lymphocyte cell adhesion molecule
BRV	baboon orthoreovirus
CAB	crucian carp blastulae embryonic cell line
CCM	CSV-containing media
CCO	channel catfish ovary cell line
cDNA	complementary DNA
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
cFLIP	cellular FLICE inhibitory proteins
CHSE-214	Chinook salmon embryo cell line
CHX	cycloheximide
CEP	circulating endothelial cells
CLC	carp peripheral blood leukocyte cell line
cox	cyclooxygenase
CRV	catfish reovirus
CSV	chum salmon reovirus
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
D-PBS	Dulbecco's phosphate buffered saline
DRAF	dsRNA-activated factor
DRBM	dsRNA binding motif
dsRNA	double stranded RNA
DTT	dithiothreitol
EHNV	epizootic haematopoietic necrosis virus (EHNV)
EPC	epithelioma carp papulosum cell line (chapter 1)
EPC	endothelial progenitor cells (chapter 7)
EROD	7-ethoxyresorufin-O- deethylase
FADD	Fas-associated death domain
FBS	fetal bovine serum
FG-9307	Japanese flounder gill cell line
GCHV	grass carp hemorrhagic virus
gDNA	genomic DNA
GMCL	goldfish macrophage cell line
HA	homotypic aggregation
H_2DCF	2',7'-dichlorodyhydrofluorescein
HINAE	hirame natural embryo cell line

HRV	hirame rhabdovirus
hsp	heat shock protein
IAP	inhibitors of apoptosis
ICAM	intercellular adhesion molecule
ICCM	interferon containing conditioned media
IFN	interferon
IHNV	infectious haematopoietic necrosis virus
IL	interleukin
IPNV	infectious pancreatic necrosis virus
IRE	interferon regulatory element
IRF	interferon regulatory factor
ISAV	infectious salmon aneamia virus
ISG	interferon stimulated gene
ISRE	interferon stimulated response element
Jak	Janus kinase
L-15	Leibovitz's media
LCDV	lymphocystic disease virus
LFA	lymphocyte function associated molecule
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MPC	mesenchymal stem cells
MRV	mammalian orthoreovirus
NBV	Nelson Bay orthoreovirus
NF-κB	nuclear factor-κB
NK cell	natural killer cell
2',5' OAS	2',5' oligoadenylate synthetase
ORV	orthoreovirus
PB	peripheral blood
PBLE	American eel peripheral blood leukocyte cell line
PCR	polymerase chain reaction
pi	post infection
PKR	dsRNA-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
Poly IC	poly inosinic:poly cytidylic acid
RNA	ribonucleic acid
RNase	ribonuclease
RRV	reptilian orthoreovirus
RTG-2	rainbow trout gonadal fibroblast-like cell line
RT-PCR	reverse transcription-PCR
RTS11	rainbow trout macrophage-like cell line
SHK-1	Atlantic salmon head kidney macrophage cell line
SHRV	snakehead rhabdovirus
SPC	smooth muscle progenitor cells
ssRNA	single stranded RNA
STAT	signal transducers and activators of transcription
SVCV	spring viremia of carp virus
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TNF-α	tumor necrosis factor- α
TO	atlantic salmon head kidney cell line
VCAM	vascular cell adhesion molecule
VHSV	viral hemorrhagic septicemia virus

vig	viral induced gene
VNNV	viral nervous necrosis virus
ZF4	zebrafish embryo fibroblast cell line
ZFL	zebrafish liver cell line
ZLE	zebrafish liver epithelium cell line

Chapter 1

General Introduction

GENERAL INTRODUCTION

The overarching goal of this thesis is to explore selected antiviral mechanisms at the cellular level, using fish cell lines. Relatively little is known about antiviral defenses in fish. Yet in the long-term, understanding antiviral mechanisms at all levels of organization could improve aquaculture and possibly aid in the management of fisheries. This section of the thesis gives an overview of knowledge about viruses in fish biology and reviews briefly the literature on the molecular and cellular biology of antiviral mechanisms in vertebrates, primarily mammals. The value of cell lines in discovering these mechanisms is discussed and recent developments in establishing monocyte/macrophage cell lines from fish is reviewed. The more specific goals are presented at the end of this general introduction.

1.1. FISH AND VIRUSES

1.1.1. Aquaculture

Aquaculture represents the fastest growing sector of animal husbandry in North America and the world (Meyer, 1991; Gudding et al., 1999). In fact, production from Canada's aquaculture industry has been steadily increasing over the past ten years (Doubleday, 2001). This growth can be attributed to declines in wild stock harvests and increases in global consumption of fish and shellfish (Meyer, 1991; Heppell and Davis, 2000). Disease causes the most significant losses in aquaculture facilities, with viral infections having especially devastating effects, up to 100% mortality (Meyer, 1991). In many cases, the only recourse is to quarantine and destroy infected stock (Munday and Nakai, 1997). Canada has experienced severe losses on both coasts due to infectious salmon anemia virus (ISAV) outbreaks in New Brunswick (Kibenge et al., 2000; Bouchard et al., 1999) and infectious haematopoietic necrosis virus (IHNV) outbreaks in British Columbia (St Hilaire et al., 2001). Wild stocks and aquaculture facilities in Europe, Scandinavia, regions of the Indo-Pacific and Mediterranean as well as the United States of America have also been affected by serious viral infections (King et al., 2001; Mortensen et al., 1999; Meyers et al., 1999; Iwamoto et al., 2001).

High mortality rates and severe economic losses emphasize the need for a better understanding of the fish response against viruses in order to better combat disease. As in mammals, the fish innate immune system may play a more important role in combating viral infections compared to the adaptive immune system. This is because innate mechanisms are non-specific, acting against viruses regardless of type, and are always present thus having no time lag between infection and immune response. This contrasts with the adaptive immune response, where in salmonids for example, it takes 4-6 weeks at optimal temperatures to produce antibodies against a new pathogen. In the case of many viral pathogens, the infected fish would be dead before specific antibodies could be

produced if it were dependent solely on the adaptive immune system (Ellis, 2001). Physical barriers, such as mucous and integument, as well as complement proteins are examples of constitutively expressed innate immune responses used by fish to combat viral infections (Lorenzen and La Patra, 1999; Dorson and Torchy, 1979). In addition to these obstacles, there is evidence that the teleost fish immune system responds to viral infection in a similar fashion to mammalian responses, namely through virus induced type I interferon production and subsequent induction of interferon stimulated genes. Thus, understanding type I interferon expression and/or its effects in fish may allow for new insights into fish antiviral mechanisms and suggest new avenues to pursue to combat viral infections.

1.1.2. Fish Viruses

Teleost fish are susceptible to a wide variety of animal viruses (Figure 1-1). Fish mortality due to viral infection is difficult to monitor in wild stocks due to insufficient monitoring techniques and procedures. Most reported cases of virus epizootics in wild fish stocks are based on chance encounters by sparse monitoring efforts or speculation when fish harvests are less than expected. Fish mortality induced by viruses is more easily demonstrated in aquaculture, where fish are monitored regularly and are localized within one area. For example, in 1995, two commercial hatcheries in Norway experienced acute mortality of their larval and juvenile fish stocks. In both cases the mortality rates reached 100%, resulting in the loss of over one million fish larvae and juveniles. It was determined from immunofluorescence, immunohistochemistry and transmission electron microscopy techniques that the fish died from a viral nervous necrosis virus (VNNV) infection (Grotmol et al., 1997). Both viral hemorrhagic septicemia virus (VHSV) and VNNV have caused acute mortality in juvenile and larval stages of economically important teleost fish species.

The realities of stressful and high-density conditions in aquaculture favour the transmission of viral infections. Negligence with hygiene regulations and failure to monitor food for viral contamination also contribute to outbreaks in hatcheries and fish farms (Munday and Nakai, 1997; Stone et al., 1997). Evidence also suggests that infected farmed fish may have been responsible for infecting healthy, wild fish stocks. In 1999, farmed salmon infected with ISAV escaped from their net-pens and were found attempting to spawn in the Magagauadavic River, New Brunswick. The next year, returning wild salmon tested positive for ISAV for the first time, and presently the virus can now be found in fry from the Magagauadavic River (Doubleday, 2001).

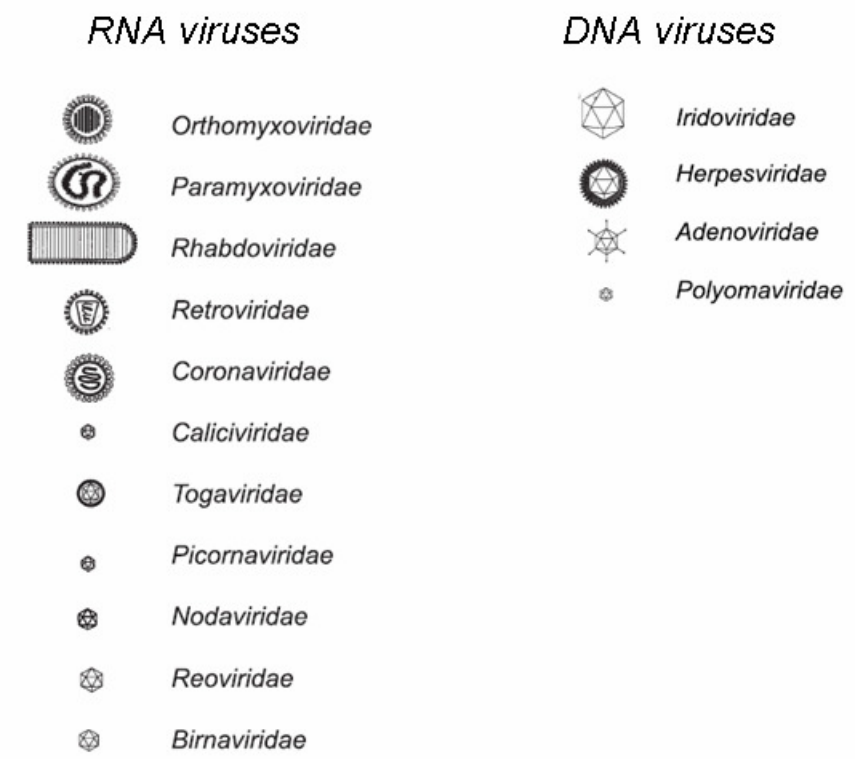

Figure 1-1. A list of animal virus families that are able to infect teleost fish.
(Adapted from Essbauer and Ahne, 2001).

Viruses are able to infect host cells *via* pH dependent or independent pathways. Some viruses enter the cell through endosomes; the low pH in the endosome facilitates viral genome entry into the cytoplasm in a pH dependent manner. Other viruses are able to fuse their membranes with the host cell membrane directly, using a pH independent mechanism (Kielian and Jungerwirth, 1990). In salmon, ISAV has been shown to enter cells *via* sialoglycoprotein residues on the cell surface using a pH-dependent mechanism. There have been attempts to identify the sialic residues recognized by ISAV in order to develop a resistant salmon strain (Eliassen et al., 2000).

Many viral infections result in death; however, in some cases a persistent infection, known as a 'carrier state', is established. Fish in this carrier state demonstrate no symptoms of the disease and have no detectable virions in their tissues (Amend, 1975); however, they are able to shed high concentrations of viruses into the water column (Kocan et al., 1997) or into their ovarian or seminal fluid during spawning (Engelking and Leong, 1981), thus infecting other fish. It has been suggested that Pacific herring may act as a reservoir of VHSV and was considered the primary source of the virus found in returning salmon and cod in Washington, USA in 1988 (Stone et al., 1997). This idea

of a carrier fish can explain how geographically separated fish species can be affected by the same virus, through a highly migratory carrier fish species (Curtis et al., 2001).

1.2. ANTIVIRAL DEFENSES

Antiviral defenses are multiple and interacting, two of which are the interferon system and apoptosis, which are reviewed here. Also included is a literature summary on the phenomenon of homotypic aggregation (HA), which might be part of antiviral defenses. The interaction between these systems comes about through shared components of underlying signal transduction pathways. One such component is the double-stranded RNA dependent protein kinase, PKR.

1.2.1. Interferon system

The interferon system consists of the interferons (IFNs), the signaling pathways triggered by IFNs binding their receptors, the transcription factors activated by these pathways, the genes whose expression is altered as a result of transcription factor activation, and finally the change in cellular function. The function that led to the discovery of the IFNs was their capacity to establish an 'antiviral state' in infected cells as well as neighbouring uninfected cells (Figure 1-3) (Goodbourn et al., 2001). Subsequently, IFNs were found to have immune-modulating functions as well as the ability to regulate cell proliferation and differentiation. IFNs are divided into two classes, type I and type II. Classically, type I IFNs are involved in antiviral mechanisms, whereas type II IFNs promote antibacterial immunity (Decker et al., 2005). Therefore, only type I IFNs are discussed here.

Type I interferon production

Type I IFN in Mammals

There are seven subtypes of type I interferons (IFN), IFN-α, IFN-β, IFN-δ, IFN-κ, IFN-ε, IFN-ω , and IFN-τ (Pestka et al., 2004). Type I IFN-like molecules have been identified in a range of organisms, from bony fish to reptiles to birds and mammals (Roberts et al., 1998). The cellular source of type I interferon is subtype dependent. IFN-β is mostly produced by non-hematopoietic cells namely fibroblasts, while IFN-α and IFN-ω are mainly produced by hematopoietic cells. IFN-τ is produced in high quantities in pre-implantation embryos of some ruminants, IFN-κ is produced by keratinocytes, and IFN-ε plays a role in the reproductive function of placental mammals, while IFN-δ is associated with porcine trophoblasts (Pestka et al., 2004). All type I IFNs are secreted monomers proteins (Sen, 2001; Roberts et al., 1998). IFN -α and -β have been the most extensively studied of the type I IFNs because of their antiviral characteristics. In fact, knockout mice lacking IFN-α/β receptors are unable to mount an efficient response against virus infections (Hwang et al., 1995;

Muller et al., 1994). Type I interferons have been shown to inhibit every stage of viral replication. This includes viral entry and uncoating, transcription, RNA stability, initiation of translation, maturation, assembly and release (Stark et al., 1998).

Interferon induction by double-stranded (ds) RNA in mammals

The induction of type I IFN expression is complex and cell-type specific. Generally, an infecting virus begins replicating its genome and sometime during this replicative cycle dsRNA is produced (Figure 1-2) (Jacobs and Langland, 1996). Viruses with dsRNA genomes, such as the reoviruses, on a whole do not allow their genomes to leave the protective inner viral capsid throughout their life cycle. However, small amounts of dsRNA can be incorrectly packaged or uncoated, thus being exposed to dsRNA-sensitive host proteins. With ssRNA viruses, their genomes are transcribed in both sense and antisense directions, causing a dsRNA replicative intermediate. Finally, DNA viruses can produce dsRNA late in an infection, when transcription fails to terminate at the end of a gene. Thus mRNAs are produced that contain complementary sequence from incorrectly transcribed gene sequences read in sense and antisense directions. These mRNAs can complex with each other and produce dsRNA. It is suggested that retroviruses do not make dsRNA; however, some retrovirus genomes have been found to form a secondary structure that is believed to interact with host dsRNA-binding proteins (Jacobs and Langland, 1996). Although these events can be rare, the potency of the molecule allows for dramatic antiviral effects.

In human fibroblasts viral dsRNA induces the assembly of an 'enhancesome', composed of transcription factors NF-kB, interferon regulatory factor-3 (IRF-3) and the ATF-2/c-Jun heterodimer (Goodbourn et al., 2001). This enhancesome controls the transcription of IFN-β and IFN-α4 (Thanos, 1996). It is not clear how dsRNA directly induces the formation of the enhancesome (Goodbourn et al., 2001). IFN-β is then secreted and can act in an autocrine fashion to stimulate the expression of IRF-7, a transcription factor controlling IFN-α synthesis. In fibroblasts, the synthesis of IFN-α requires IFN-β expression; however, this is not the case for human leukocytes, where IFN-α can be expressed independent of IFN-β (Erlandsson et al., 1998). After this early type I interferon secretion, a second wave of cytokines follow, these include: tumor necrosis factor-α (TNF), interleukin (IL)-6, IL-12 and IFN-γ (Sen, 2001).

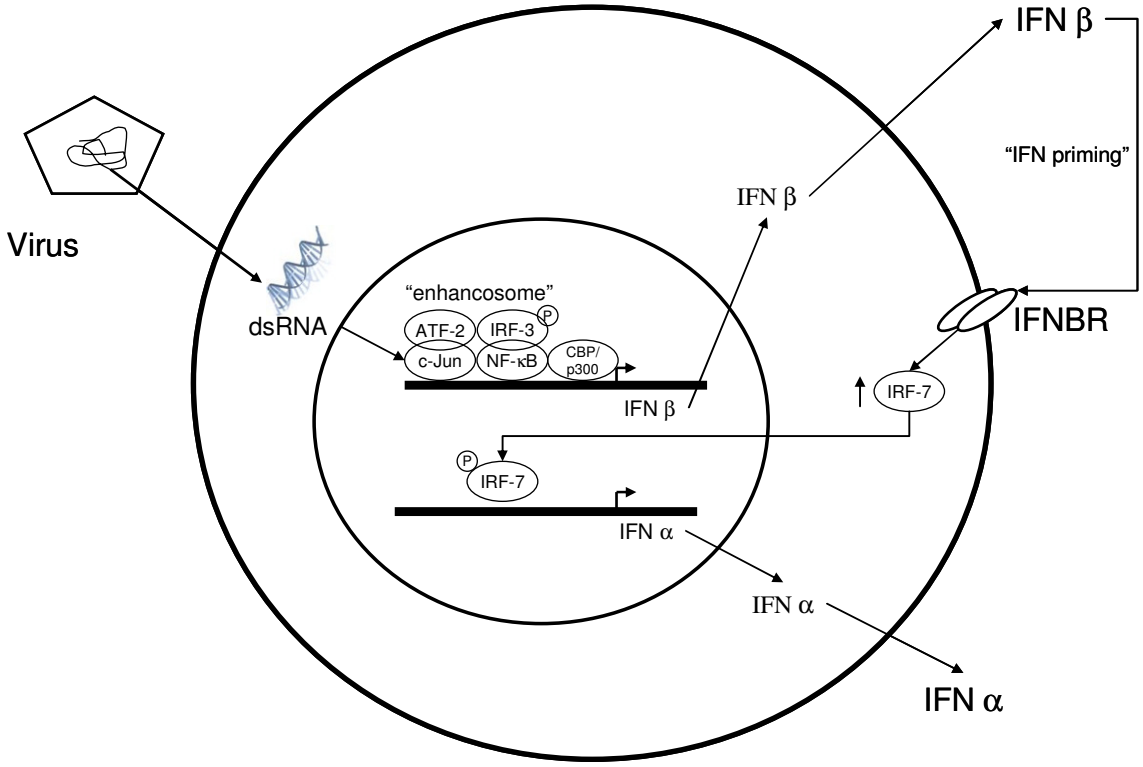

Figure 1-2. A diagrammatic summary of early type I IFN production by the host cell in response to viral infection.

The pathway is a summary of what is known of mammalian type I interferon induction and expression (Adapted from Sen, 2001). A viral infection causes the production of dsRNA sometime during its replicative cycle. This dsRNA triggers the activation of transcription factors and formation of the 'enhanceosome' complex which includes an ATF-2/c-Jun heterodimer, IRF-3 and NF-κB. The enhanceosome recruits the CBP/p300 coactivator to the pre-initiation complex. This complex causes the production of IFN-β, which can act in an autocrine fashion to prime the cell to make a new set of IFNs, known as "IFN priming". IFN- β binds to its cognate receptor, and through a cascade of events triggers the expression of IRF-7, which can be activated and act as a transcription factor to induce expression of a new set of IFN, IFN-αs. Abbreviations: **dsRNA** – double stranded RNA, **IFNα/β** - interferon α/β, **INFBR** – interferon β receptor, **IRF**-interferon regulatory factor.

Type I IFN in Fish and effects of dsRNA

Soon after Isaacs and Lindemann's discovery in 1957, interferon-like antiviral activity was discovered in ectothermic animals including fish. These early experiments detected interferon activity *in vitro* following virus infection using cell lines derived from fathead minnow and blue striped grunt (Beasley and Sigel, 1967; Beasley et al., 1966; Gravell and Malsberger, 1965; Oie and Loh, 1971). *In vivo* studies were also performed and antiviral activity was detected in rainbow trout serum (De Kinkelin and Dorson, 1973; Dorson et al., 1975). More recently it was found that poly

inosinic: poly cytidylic acid (poly IC), a synthetic dsRNA, could also induce an antiviral, 'interferon'-like response in fish (Eaton, 1990).

Although interferon activity had been clearly demonstrated, it has taken almost 35 years to convincingly isolate, sequence and characterize interferon in fish. In 1975, a partially purified protein with physical, chemical, and biological properties of interferon was isolated from infectous pancreatic necrosis virus (IPNV) treated RTG-2, a rainbow trout gonadal cell line (De Sena and Rio, 1975). In 1993, the first fish interferon sequence was reported from flatfish (*Paralichthys olivaceus*) (Tamai et al., 1993). Unfortunately, this putative interferon sequence showed no similarity to any other known interferon, and did not contain any of the highly conserved residues required for its function in mammals. It was later found that two-thirds of the clone showed significant similarity to filamentous phage sequence (Magor and Magor, 2001). Ten years later, interferon sequences were reported from zebrafish (*Danio rerio*) (Altmann et al., 2003), channel catfish (*Ictalurus punctatus*) (Long et al., 2004), Atlantic salmon (*Salmo salar*) (Robertsen et al., 2003), spotted green pufferfish (*Tetradon nigrovirides*) (Lutfalla et al., 2003), and a fragment from rainbow trout (*Oncorhynchus mykiss*; unpublished, AJ582754) and Japanese pufferfish (*Takifugu rubripes*; unpublished, CAE47314). All published interferon clones demonstrated antiviral activity (Table 1-1).

Table 1-1. A summary of the antiviral activity of isolated interferon from teleost species.

Species Isolated	Cells tested	IFN-stimulus	Antiviral activity measured	Reference
Danio rerio, zebrafish	Zebrafish embryo fibroblast (ZF4) transfected with zfIFN	Snakehead rhabdovirus (SHRV)	Plaque reduction in transfected cells	(Altmann et al., 2003)
Ictalurus punctatus, channel catfish	Channel catfish ovary (CCO)	Poly IC (50µg/mL), UV – inactivated catfish reovirus (CRV), recombinant cfIFN	Conditioned media and recombinant cfIFN protected CCO against CRV	(Long et al., 2004)
Salmo salar, Atlantic salmon	TO (Atlantic salmon head kidney) and CHSE-214 (Chinook salmon embryo)	Recombinant ssIFN	Protected cells against IPNV	(Robertsen et al., 2003)
Tetradon nigrovirides, spotted green pufferfish	Cephalic kidney cells (primary culture)	Recombinant tnIFN	Induced ISG, *Mx*, as detected by RT-PCR	(Lutfalla et al., 2003)

Interestingly, there was very little similarity between the zebrafish interferon sequence and other known mammalian and avian sequences; however, the zebrafish interferon sequence did

contain two cysteine residues and a phenylalanine that are important for interferon function in mammals (Altmann et al., 2003). Also, secondary structure analysis of Atlantic salmon IFN suggests that fish IFN possess 5 alpha helixes. This suggests that fish type I IFN have a similar conformational shape as mammalian type I IFN in spite of low sequence similarity (Robertsen et al., 2003). With the identification of interferon in fish, the pathways initiated by this potent cytokine can be identified.

Type I Interferon Signaling Pathways

Signaling pathways in mammals

The antiviral effects of type I IFNs are initiated by the binding of interferon to its cognate receptor found on the surface of all nucleated cells (Sen, 2001). The IFNα/β signaling pathway in mammals involves five major steps (Figure 1-3). IFN binding causes dimerization of the IFN receptor (1). This receptor association triggers signaling through the Janus kinase (Jak)/Signal transducers and activators of transcription (STAT) pathway by activating Janus kinases, Jak1 and Tyk2 (2). These tyrosine-kinases phosphorylate STAT1 and STAT2, which are associated with the IFN receptor, leading to their activation and dimer formation (3). The activated STAT 1-2 heterodimers translocate to the nucleus (4) and associate with p48 (IRF-9) to form ISGF3, a transcription factor which binds to interferon-stimulated response element (ISRE) sequences in the promoter regions of interferon stimulated genes (5; ISGs) (Stark et al., 1998). Type I interferons generally induce the same set of genes within the same cell type. It is these ISGs that accumulate in the target cell, establishing an 'antiviral state'.

IRF-1 was the first interferon-regulatory factor (IRF) discovered, since then nine mammalian IRFs have been identified. All IRFs are able to bind to a consensus DNA element, IRE (interferon regulatory element), and modulate transcription (Sen, 2001). In fact, an IRE is similar to an ISRE; therefore, IRFs such as IRF-1 can modulate transcription of type I IFNs and ISGs (Matsuyama et al., 1993; Ruffner et al., 1993), representing a second wave of transcription after Jak/STAT signaling (Levy and Garcia-Sastre, 2001; Stark et al., 1998). Expression of many important ISGs can be modulated by IRF-1, including: 2', 5' oligoadenylate synthetase (OAS) (Harada et al., 1998; Reis et al., 1992) and dsRNA-dependent protein kinase (PKR) (Kirchhoff et al., 1995; Samuel et al., 1997).

IRF-1 plays many roles besides IFN-stimulated antiviral mechanisms. It can inhibit cell growth, (Kirchhoff et al., 1993; Kirchhoff et al., 1996) perhaps through upregulation of PKR (Kroger et al., 2002). This characteristic of IRF-1 may also be associated with its tumor suppressive capabilities (Tanaka et al., 1994). IRF-1 can mediate DNA damage induced apoptosis in lymphocytes (Tamura et al., 1995). Finally, IRF-1 can modulate immune responses not only through upregulation of type I

interferon and important antiviral proteins, but also by affecting NK cell development (Ohteki et al., 1998; Ogasawara et al., 1998), reducing class I MHC cell surface expression (White et al., 1996), and being a requirement for TH1 responses *in vivo* (Lohoff et al., 1997; Taki et al., 1997).

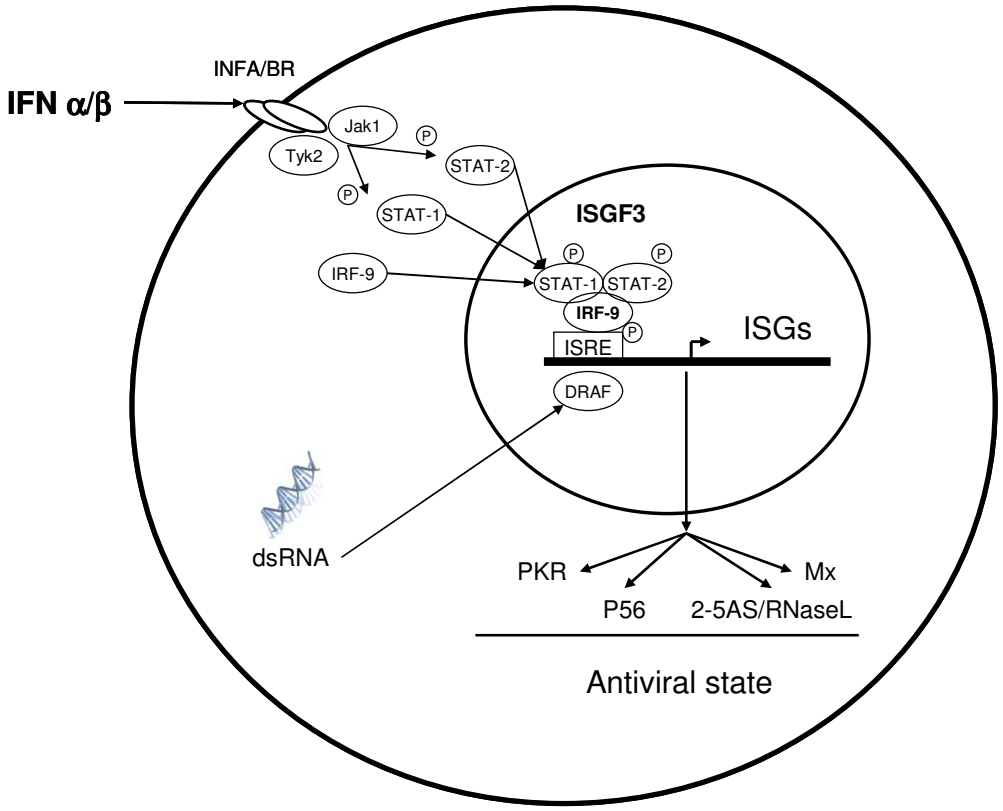

Figure 1-3. A diagrammatic summary of the early effects of type I IFN to create an antiviral state in a host cell.

The binding of a type I IFN to its cognate receptor causes the dimerization of the JAKs, Jak1 and Tyk2, which are transphosphorylated and activated. They in turn phosphorylate STAT1 and STAT2, which dimerize and migrate to the nucleus, where they associate with IRF-9 to form the transcription factor complex, ISGF3. ISGF3 binds to the ISRE in the promoter region of the ISGs, activating transcription. An interferon independent pathway, involving dsRNA and DRAF, can also result in expression of ISGs. In either case, the ISG proteins accumulate within the cells in an inactive form, creating an antiviral state. Abbreviations: **IFN** - interferon, **INFA/BR** - interferon α/β receptor, **JAK** - Janus kinases, **STAT** - signal transducer and activator of transcription, **IRF** - interferon regulatory factor, **ISRE** - interferon stimulated response element, **ISG** - interferon stimulated gene, **DRAF** – dsRNA-activated factor, **PKR** – dsRNA-dependent protein kinase, **2-5AS** – 2', 5' oligoadenylate synthetase.

IRF-2 negatively regulates transcription of ISGs. It does this by competing with IRF-1 for the same DNA-binding sites (Harada et al., 1989; Yamamoto et al., 1994). Compared to IRF-1, IRF-2 has a much longer half-life (Watanabe et al., 1991), and is expressed after IRF-1 therefore

functioning to control IRF-1 stimulated gene expression (Harada et al., 1994). IRF-2 may also play a role in inhibiting the expression of ISGs in healthy cells in the absence of IFN (Stark et al., 1998).

There are other negative modulating factors that attenuate interferon signaling, these factors are also interferon-stimulated proteins. IRF-8 is able to bind ISREs, suppressing transcription and similarly to IRF-2, IRF-8 is believed to prevent inappropriate activation of ISGs under normal conditions. The SOCS/JAB/SSI family of ISGs inhibits signaling by binding and inhibiting Jaks. STAT1 can also be regulated by tyrosine phosphatase mediated dephosphorylation (Starr and Hilton, 1999).

It is interesting to note that in mammals, dsRNA has been shown to induce ISG transcription independent of interferon induction. This has been studied in cell lines that do not produce interferon or are not sensitive to its actions. In these studies, transcription factors such as DRAF, VAF and VIF are activated and induce transcription of some ISGs (Sen, 2001). In the case of DRAF1 (dsRNA-activated factor1), two of its components are IRF-3 and CREB-binding protein (CBP)/p300 (Weaver et al., 1998). Since a large number of infecting viruses inhibit interferon expression, (Levy and Garcia-Sastre, 2001; Goodbourn et al., 2001) the ability of an infected cell to express ISGs without interferon may be an effective back-up antiviral mechanism. So far, nothing is known of this alternative pathway in fish.

Signalling pathways in fish

Very little is known of the IFN signaling pathway in fish. The components of the pathway seem to be present; Janus kinases have been shown to be active in fish (Rycyzyn et al., 1998b; Evans et al., 1999), as well as STATs (Rycyzyn et al., 1998a). Jak1 and Tyk2 have been identified in pufferfish (*T. fluviatilis*) (Leu et al., 2000), and STAT 1 has been cloned from zebrafish and crucian carp (Zhang and Gui, 2004; Oates et al., 1999). Studies have shown that STAT1 is upregulated in rainbow trout following IHNV infection (Hansen and La Patra, 2002), and in carp following poly IC and grass carp haemorrhagic virus (GCHV) infection (Zhang and Gui, 2004), as well as being able to rescue type I IFN signaling mechanisms in STAT1-negative cells (Oates et al., 1999). These results suggest that fish have a functioning Jak/STAT pathway similar to that observed in mammals.

IRFs have been identified in rainbow trout (*O. mykiss*) (Collet et al., 2003), Japanese flounder (*P. olivaceus*) (Yabu and Hirose, 1998), crucian carp (*C. auratus*) (Zhang et al., 2003a) and pufferfish (*Fugu rubripes*) (Richardson et al., 2001). In Japanese flounder an IRF-like gene was upregulated in response to infection with Hirame rhabdovirus (HRV) and *Edwardsiella tarda* (Yabu and Hirose, 1998). More recently an IRF-1 like molecule was shown to induce an antiviral state, as supernatants from transfected cells expressing JF IRF-1 protected cultures exposed to VHSV and

HRV by inhibiting cytotoxicity (Caipang et al., 2005). An IRF-1-like molecule was identified in pufferfish tissue (Richardson et al., 2001). In rainbow trout both IRF-1 and IRF-2 have been cloned, and their transcripts were constitutively expressed in head kidney, gill, and spleen; however, expression was upregulated *in vivo* in response to VHSV infection, and *in vitro* in response to poly IC (Collet et al., 2003). IRF-3 has yet to be identified in any fish species. It has been suggested that IRFs in fish function similar to those in mammals; however, the exact mechanisms of action have yet to be fully elucidated (Collet and Secombes, 2002).

ISGs and the Antiviral State

The primary purpose of type I interferons is to stimulate the expression of ISGs, which in turn confer an antiviral state within uninfected cells (Figure 1-3). Interferon treatment can alter the expression of over 1000 different genes (Der et al., 1998). These interferon inducible factors tend to either limit virus replication directly or regulate cell cycle and cell death (Goodbourn et al., 2001). Programmed cell death or apoptosis, which is stimulated by some ISGs, is considered a strategy to control viral replication (Everett and McFadden, 2001). Many IFN stimulated proteins are enzymes that are expressed in an inactive form until exposed to dsRNA, ensuring an antiviral state that remains dormant and therefore harmless until the cell is infected (Jacobs and Langland, 1996). ISG expression depends on cell type and IFN type; they include enzymes, chemokines, antigen presentation proteins, transcription factors, heat shock proteins and apoptotic proteins (Sen, 2001). The best-characterized proteins that control viral replication in mammals are dsRNA-dependent protein kinase (PKR), Mx proteins, and the 2'-5' oligoadenylate synthetase (OAS)/RNaseL pathway.

Only a handful of ISGs have been identified and characterized in fish. These include Mx proteins (Leong et al., 1998), IRF-1 and -2 (Collet et al., 2003), virus induced gene-1 (vig-1) and vig-2 (Boudinot et al., 1999; Boudinot et al., 2001), a host of uncharacterized *vig*s whose functions are a matter of speculation (O'Farrell et al., 2002), as well as a PKR-like molecule, PKZ (Rothenburg et al., 2005; Hu et al., 2004a). One valuable study measured changes in gene expression following viral infection using suppressive subtractive hybridization (O'Farrell et al., 2002). It was found that VHSV infected rainbow trout leukocytes expressed eight full-length genes not found in uninfected cells. Four genes were identified as interferon responsive genes, three were similar to chemo-attractant molecules and two others had nucleic acid binding domains. This study clearly demonstrated that viral infection in fish induces the expression of many different genes, some of which are related to interferon.

PKR

In mammals, double-stranded RNA dependent protein kinase (PKR) plays an important role in the antiviral responses of innate immunity. In humans, PKR is a 551 amino acid protein, with an amino-terminal regulatory domain containing two dsRNA binding motifs (DRBM) and a carboxy-terminal catalytic domain containing eleven conserved kinase subdomains of the eIF2 α kinase family (Figure 1-4) (Jagus et al., 1999).

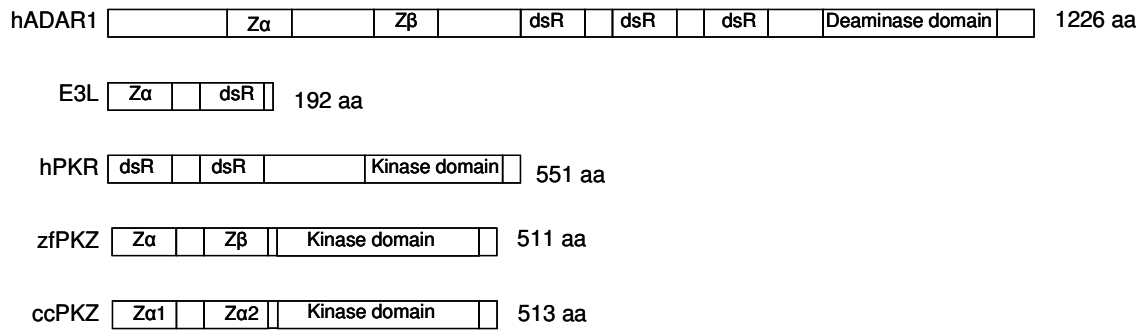

Figure 1-4. A diagrammatic comparison of proteins containing dsRNA or Z-DNA binding domains.

Fish PKZ proteins, both zebrafish (zfPKZ) and crucian carp (ccPKZ), contain two Z-DNA binding motifs (Zα,β) and a kinase domain. Human PKR is of similar size but has two dsRNA binding motifs (dsR) instead of the Z-DNA binding domains. The vaccinia viral protein, E3L, contains both a Zα and a dsR, as does the human dsRNA adenosine deaminase 1 (hADAR1) (Rothenburg et al., 2005; Hu et al., 2004a). Abbreviations: **aa** amino acid

This serine/threonine kinase is activated through autophosporylation and dimerization upon binding to dsRNA (Sen, 2000). Activated PKR phosphorylates many endogenous substrates, including serine 51 of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (Levin and London, 1978). Phosphorylated eIF2α sequesters eIF2B and prevents the recycling of eIF2-GDP to eIF2-GTP, ultimately resulting in global inhibition of translation (Lengyel, 1993). PKR has also been associated with activating NF-κB and IRF-1, transcription factors that control expression of important antiviral genes (Clemens, 1997; Kirchhoff et al., 1995). It is through these substrates that PKR can inhibit viral replication by blocking translation, (Koromilas et al., 1992; Clemens, 1997) inhibiting cell proliferation, (Chong et al., 1992; Jagus et al., 1999) and triggering apoptosis (Srivastava et al., 1998; Der et al., 1997; Wada et al., 1995; Takizawa et al., 1996; Yeung et al., 1999).

Though one of the most extensively studied interferon stimulated proteins in mammals (Sen, 2000), very little is known of PKR in teleosts. Indirect evidence suggests the presence of an antiviral

kinase similar to PKR in mammals. In one study, epizootic haematopoietic necrosis virus (EHNV)-induced apoptosis in the epithelioma carp papulosum (EPC) cell line was blocked by 2-aminopurine, a PKR-specific inhibitor. This suggests the involvement of PKR in the induction of apoptosis during a viral infection, similar to its role in mammals (Essbauer and Ahne, 2002). Other evidence for PKR activity in fish includes elevated levels of phosphorylated eIF2α in RTG-2 and ZFL, a zebrafish liver cell line, upon treatment with poly IC or infectious pancreatic necrosis virus (IPNV) (Garner et al., 2003). In this study, the authors suggest that as in mammals, fish possess the key components of the interferon induced PKR pathway (Garner et al., 2003). Recently a putative PKR-like molecule has been identified in crucian carp (*Carassius auratus*) (Hu et al., 2004a) and zebrafish (*Danio rerio*) (Rothenburg et al., 2005). In both reports the PKR-like protein contained a kinase domain similar to the eIF2α family of kinases; however, neither protein contained a DRBM, instead they had two Z α domains which bind dsDNA and RNA in the left-handed Z conformation. Designated PKZ, this kinase was suggested to be the teleost fish form of PKR because it was able to block protein synthesis, it contained a conserved kinase domain, and its low constitutive expression was strongly induced by both dsRNA and viruses (Rothenburg et al., 2005). It is unclear why fish PKR would contain Z-DNA binding motifs, although it has been shown that Z α domains of the enzyme ADAR (dsRNA adenosine deaminase) are able to bind dsRNA by converting it from A to Z-conformation (Brown et al., 2000), and thus may be the case for PKZ. It is also possible that the differences in binding domains reflect differences in fish viruses compared to those that challenge mammals (Rothenburg et al., 2005).

Mx

Mx proteins were first identified from orthomyxovirus resistant mice strains (Lindenmann, 1962; Isaacs, 1963). Two Mx proteins have been identified in humans (Staeheli and Haller, 1985; Aebi et al., 1989), three in rat (Meier et al., 1988) and two in mice (Staeheli and Sutcliffe, 1988). At least one Mx protein from each of these animals demonstrates antiviral activity against influenza virus (Pavlovic and Staeheli, 1991). Mx proteins have since been found in many other mammalian species (Horisberger and Gunst, 1991) as well as birds (Bazzigher et al., 1993; Bernasconi et al., 1995) and fish (Leong et al., 1998). Mx proteins are related to the dynamin superfamily, which are high molecular weight GTPases found in yeast, plant and animal cells (Pavlovic and Staeheli, 1991) and are interferon inducible (Haller et al., 1980). In mice, Mx1 interferes with the primary transcription of influenza virus by disturbing polymerase function, while human MxA blocks a post-transcriptional step (Haller et al., 1998). It has been suggested that Mx antiviral activity is associated with its intracellular localization (Pavlovic and Staeheli, 1991) and its ability to hydrolyze GTP

(Melen and Julkunen, 1994; Ponten et al., 1997); however, the exact antiviral mechanism of Mx proteins has not been conclusively shown and may vary between species.

The first fish Mx protein was isolated from fresh water perch, *Perca fluviatilis,* and was shown to be inducible by dsRNA and viral infection (Staeheli et al., 1989). Since then Mx proteins have been identified in many fish including: rainbow trout (*O. mykiss*) (Trobridge and Leong, 1995; Trobridge et al., 1997b), Atlantic salmon (*S. salar*) (Robertsen et al., 1997), halibut (*Hippoglossus hippoglossus*) (Jensen and Robertsen, 2000), Japanese flounder (*Paralichthys olivaceus*) (Lee et al., 2000), as well as Chinook salmon (*Oncorhynchus tshawytscha*), tilapia (*Oreochromis mossambicus*), kokanee salmon (*Oncorhynchus nerka*), brook trout (*Salvelinus fontinalis*), chum salmon (*Oncorhynchus keta*) and coho salmon (*Oncorhynchus kisutch*) (Leong et al., 1998). Sequence analysis shows that the three rainbow trout Mx proteins have GTP binding sites and a dynamin family signature sequence similar to mammalian Mx proteins. The rainbow trout Mx1 and the zebrafish Mx promoter region both contain an ISRE, further evidence that fish Mx proteins are regulated similar to that in mammals (Collet and Secombes, 2001; Altmann et al., 2004).

Initially, Mx proteins were thought to not be constitutively expressed and were considered to be specifically induced by type I interferon or dsRNA both in mammals (Simon et al., 1991; Memet et al., 1991; Von Wussow et al., 1990) and fish (Nygaard et al., 2000; Leong et al., 1998) as well as by numerous viral pathogens including: IHNV (infectious hematopoietic necrosis virus) (Trobridge et al., 1997a), ISAV (infectious salmon anemia virus) (Jensen and Robertsen, 2002), SHRV (snakehead rhabdovirus), SVCV (spring viremia of carp virus) (Kim et al., 2000), HRV (hirame rhabdovirus) (Lee et al., 2000), nodavirus (Tafalla et al., 2004), IPNV (infectious pancreatic necrosis virus) (Bergan and Robertsen, 2004), CCV (channel catfish virus) (Plant et al., 2005) and GCHV (Grass carp hemorrhagic virus) (Zhang et al., 2004). When induced, Mx can be produced at high levels, up to 1% of total cytosolic proteins (Horisberger, 1995). It is these qualities that have made Mx proteins ideal for use as a marker of type I interferon expression. This has been especially useful in fish, where the study of interferon directly proved to be quite difficult (Garner et al., 2003; Kim et al., 2000). Contrasting with this classic model, it has been shown more recently that fish Mx can be induced by unmethylated CpG dinucleotides (Jorgensen et al., 2001) and *Lystonella anguillarum* lipopolysaccharide (LPS) (Acosta et al., 2004). Also, constitutive Mx expression has been reported in tissues from sea bream (*Sparus aurata*) (Tafalla et al., 2004), Atlantic salmon (*S. solar*) (Bergan and Robertsen, 2004), and Japanese flounder (*P. olivaceus*) (Lee et al., 2000) as well as in the crucian carp blastulae embryonic cell line, CAB (*C. auratus*) (Zhang et al., 2004). Until recently, the role of fish Mx proteins in inhibiting viral replication was merely speculative (Trobridge et al., 2000). However, in recent years a direct antiviral role for Mx has been described. Atlantic salmon

Mx1 protected the Chinook salmon embryonic cell line (CHSE-214) against ISAV (Kibenge et al., 2005a), and IPNV (Larsen et al., 2004). Also, Japanese flounder Mx was able to protect a transfected hirame natural embryo cell line (HINAE) from cytolysis following VHSV and hirame rhabdovirus (HRV) infection (Caipang et al., 2003).

Vig-1

Virus induced gene-1 (vig-1) was first cloned from rainbow trout (Boudinot et al., 1999). This interferon-sensitive protein shares sequence similarity with the human and mouse protein, viperin (cig5) (Boudinot et al., 2000; Chin and Cresswell, 2001). Vig-1 has since been cloned in mandarin fish (*Siniperca chuatsi*) (Sun and Nie, 2004) and has been detected using suppressive subtractive hybridization in Chinook salmon and staghorn sculpin treated with poly IC (Alonso and Leong, 2002), rhabdovirus exposed rainbow trout (O'Farrell et al., 2002) and UV-inactivated GCHV treated crucian carp cells (Zhang et al., 2003b). Vig-1 has yet to demonstrate any antiviral activity in fish.

2',5' OAS/RNaseL pathway

2',5' oligoadenylate synthetases (OASs) are a family of enzymes induced by type I IFN. When activated by dsRNA, these enzymes polymerize ATP into short 2', 5' oligoadenylates that in turn can bind and activate the latent ribonuclease, RNaseL (Carroll et al., 1996). RNaseL degrades single stranded RNA (ssRNA) resulting in inhibition of cellular and viral transcription. Using RNaseL null mice it has been demonstrated that this system is linked to IFN-stimulated antiviral effects and several apoptotic pathways (Zhou et al., 1997). Conversely, the OAS system may actually act as a modulator of intracellular IFN responses. Just recently, RNaseL null mice were shown to have increased PKR mRNA stability and enhanced IFN responses (Khabar et al., 2003). This important antiviral pathway has yet to be identified in fish.

These are examples of only a few of the over 1000 genes whose expression IFNs are able to modify (Sen, 2000). The proteins described control viral replication mainly through inhibition of viral protein synthesis and by triggering apoptosis in the host cell. Viruses in turn have developed a variety of mechanisms to elude these host antiviral proteins (Benedict et al., 2002).

Later Interferon Induced Effects

The initial innate immune response to viral infection, namely the release of interferon α/β and its resulting pathways, is just the beginning of the mammalian immune response against viruses. Interferon α/β produced by a virally infected cell not only causes an antiviral state in neighboring cells but also is involved in the inhibition of cell division, the recruitment and stimulation of NK

cells, cytotoxic T cells (CTLs) and macrophages. This cytokine is also implicated in the upregulation of MHC class I and II molecules, the induction of antibody production by B cells and stimulation of the proliferation of memory T cells (Guidotti and Chisari, 2001). Thus, the release of interferon α/β and other cytokines from a virus infected cell causes a cascade of immune responses. Macrophages present produce chemokines to attract immune cells and pro-inflammatory cytokines such as IL-12 and IL-18, which induce IFN-γ, IL-1, IL-6, and TNF-α production. INF-γ and TNF-α, produced mainly by NK cells, are powerful cytokines responsible for recruiting and activating immune effector cells, upregulating antigen processing pathways and MHC expression in infected cells and directly exerting antiviral activity (Guidotti and Chisari, 2001). It is the NK cells, and CTLs that are thought to be responsible for killing the virally infected host cell (Biron, 1999). In this way, type I interferon is vital in activating the effector cells of the innate and adaptive immune system against intracellular viral infections.

1.2.2. Apoptosis as an antiviral mechanism

General overview of apoptosis mechanisms

Apoptosis, or programmed cell death, is the systematic collapse of cellular infrastructure, genomic degradation, and cellular fragmentation, culminating in engulfment of fragments by neighbouring cells or phagocytes. Thus apoptosis is considered a mechanism for a 'clean', non-inflammatory cell death (Barber, 2000). Apoptosis is initiated, executed and regulated by caspases, a family of cysteine-based, aspartate-directed proteinases which exist as zymogens within the cell until activated (Benedict et al., 2002). There are two different cell mechanisms that lead to apoptosis (Figure 1-5), those triggered by extrinsic factors, such as members of the TNF-α cytokine family (ex. FasL, TRAIL), and those triggered by intrinsic factors, including DNA damage, cytokine deprivation, and cytotoxic drugs.

The intrinsic factors can trigger internal sensors, such as p53, which activate Bcl-2 pro-apoptotic family members (ex. Bax, Bad) causing them to assemble and form pores in the mitochondria resulting in the loss of mitochondrial membrane integrity and cytochrome c release. This released cytochrome c promotes the formation of the apoptosome, containing procaspase-9 and apoptosis-activating factor 1(Apaf-1), and the cleavage of procaspase-9 to an active caspase. Activated caspase-9 initiates the effector caspase cascade, which includes caspase-2, -3, -6, and/or –7. Extrinsic factors, known as death ligands, signal through their cognate death receptors, which are members of the TNFα superfamily of receptors. When bound, these receptors recruit adaptor proteins that contain death domains (DD) and death effector domains (DED), such as FADD (Fas-associated death domain). The DDs of the adaptor protein bind to the receptor and the DEDs bind to

pro-caspase-8 or –10. The assembly of activated receptor, adaptor protein and caspase at the cell membrane is referred to as a death-inducing signaling complex (DISC). The close arrangement of caspases in the DISC results in autocatalysis and activation of the enzyme. Activated caspase-8 and –10 trigger the effector caspase cascade as well. The final result of the effector mechanisms is cleavage of genomic DNA and cellular morphologic changes that finally lead to a non-inflammatory cell death (Figure 1-5) (Benedict et al., 2002; Clemens, 2003).

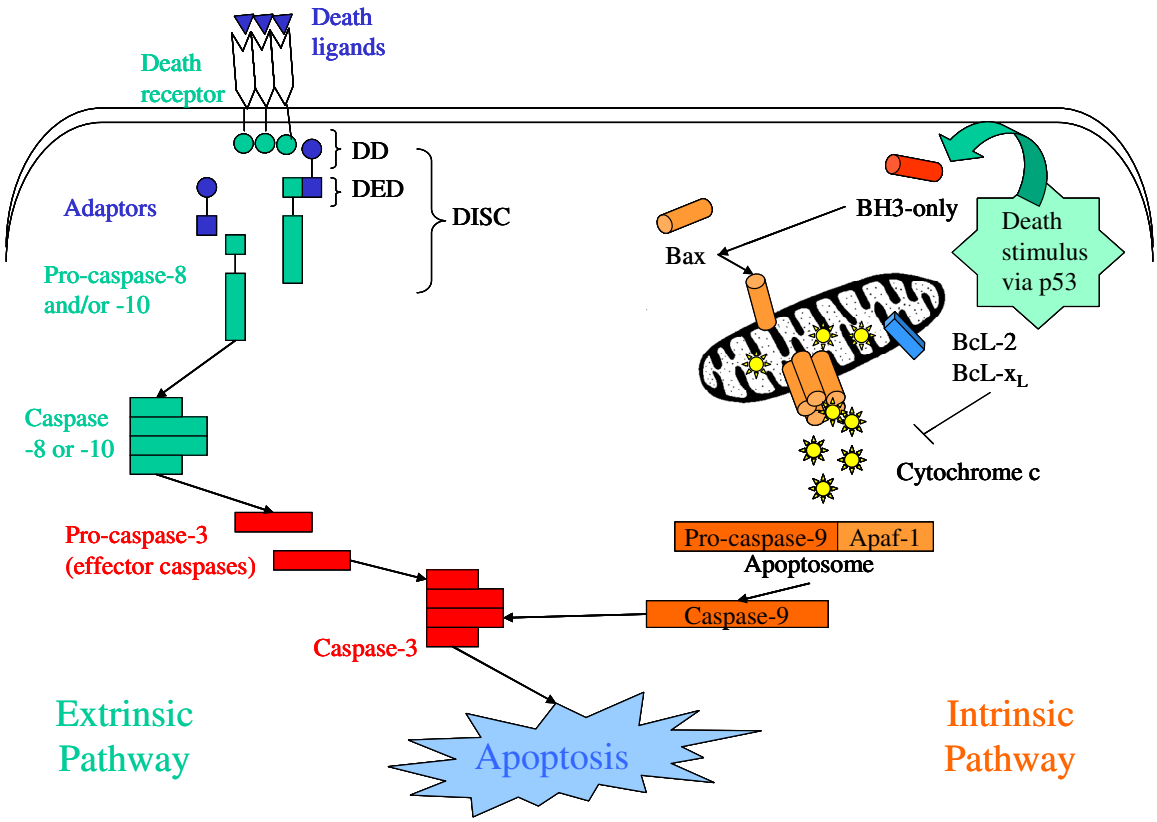

Figure 1-5. A diagrammatic summary of the extrinsic (death receptor mediated) and the intrinsic (mitochondrial mediated) apoptotic pathways.

The extrinsic and intrinsic pathways are triggered by external and internal signals respectively, and converge at the effector caspase, caspase-3. This caspase triggers numerous mechanisms within the cell which ultimately leads to programmed cell death.

Control mechanisms exist within the cell to inhibit apoptotic mechanisms. Viruses have also developed many different strategies to block apoptosis induced by viral infection (Benedict et al., 2002). One family of survival factors is the inhibitors of apoptosis (IAPs). The main role of these proteins is to block caspase activity, either by binding caspases directly thus blocking their active site, or by ubiquitinating caspases, thus tagging them for proteasome-mediated degradation (LeBlanc, 2003). Other control proteins include Bcl-2 family members that prevent cytochrome c release (Reed et al., 1996), heat shock proteins (hsps), such as hsp70 which can protect against active

caspase 3 and block apoptosome formation (Jaattela et al., 1998; Saleh et al., 2000) and cellular FLICE (caspase-8) inhibitory proteins (cFLIPs), which compete with caspase 8 for binding to the DED of the adaptor protein (Irmler et al., 1997). It is this tension between pro- and anti-apoptotic factors that ultimately dictates the fate of the cell.

Interferon induced apoptosis

At the individual cell level, viral infection can induce apoptosis. This is considered an innate immune response that inhibits viral replication and dissemination by inducing suicide of the virally infected cell. In effect, the infected cell will 'choose' to die to inhibit viral spread throughout the host and 'take one for the team' (Everett and McFadden, 1999; Teodoro and Branton, 1997; O'Brien, 1998). Both IFN-α and IFN-β have been shown to induce apoptosis, although it has been suggested that IFN-β is a more effective inducer of apoptosis compared to IFN-α (Chawla-Sarkar et al., 2001; Sanceau et al., 2000). The pathways leading to interferon-induced apoptosis have yet to be fully elucidated. It is known that both STATs and IRFs are involved (Harada et al., 1998; Mui, 1999). The intrinsic pathway is involved in IFN-induced apoptosis. IFN-α induces apoptosis through activated Bax and subsequent cytochrome c release; the involvement of caspases in initiating this response is unknown (Yanese et al., 2000; Panaretakis et al., 2003). Conversely, it has been found that IFN-α induced apoptosis in tumor cells is independent of p53 (Sangfelt et al., 1997). Even so, there seems to be more evidence indicating that mitochondrial-mediated apoptosis activation is secondary to death receptor-mediated apoptosis (Ruiz-Ruiz and Lopez-Rivas, 2002; Chen et al., 2001; Thyrell et al., 2002). It has been shown that caspase-8 activation lies upstream of the effector caspases and mitochondrial death pathways in IFN-α induced apoptosis (Thyrell et al., 2002). It has also been found that IFN can induce expression of TRAIL, a death receptor ligand, in peripheral blood thymocytes (Kayagaki et al., 1999). Although IFN may directly induce apoptosis, it may be that the most important role type I IFNs play is to sensitize cells to apoptosis, for example in response to dsRNA (Stewart et al., 1972; Stewart et al., 1973; Tanaka et al., 1998; Balachandran et al., 2000).

dsRNA induced apoptosis

dsRNA treatment itself can induce apoptosis in a variety of mammalian cell types with or without prior type I interferon exposure (Kalai et al., 2002; Kibler et al., 1997; Yeung et al., 1996; Der et al., 1997; Srivastava et al., 1998; Lee and Esteban, 1994; Lee and Esteban, 1993). DsRNA or dsRNA-activated/IFN-inducible proteins, such as PKR and 2',5' OAS, seem to induce apoptosis through the death-receptor mediated pathway. Specifically, dsRNA induces apoptosis in human Jurkat cells through caspase-8 *via* FADD-dependent and independent pathways (Kalai et al., 2002).

Also, type I IFN sensitized fibroblasts undergo apoptosis after dsRNA exposure or virus infection, through a FADD/caspase-8 dependent pathway. In this specific study it was suggested that IFN sensitization of cells to dsRNA-induced apoptosis acts by inducing expression of FADD-related proteins and increased formation of DISCs (Balachandran et al., 2000). This FADD-associated dsRNA-induced apoptosis is thought to be mediated through a PKR activated pathway (Wada et al., 1995; Takizawa et al., 1996).

Activated PKR has been implicated in the induction of apoptosis from a variety of stimuli, including live viruses and dsRNA (Srivastava et al., 1998; Der et al., 1997; Wada et al., 1995; Takizawa et al., 1996; Yeung et al., 1999). To date there are at least two proposed modes of action for PKR-induced apoptosis. Firstly, phosphorylation of eIF2α by activated PKR has been shown to be a key stimulus of apoptosis in some cell systems (Gil et al., 1999; Jeffrey et al., 2002). In fact, overexpression of the phosphorylated form of eIF2α alone can cause apoptosis in transfected NIH3T3 cells (Srivastava et al., 1998). Secondly, PKR can also mediate apoptosis by upregulating pro-apoptotic factors at the transcription level, perhaps through NF-κB activation (Gil and Esteban, 2000a).

The 2',5' OAS triggered pathway has also been implicated as a mediator in dsRNA induced apoptosis. Both 2',5' OAS and RNaseL have been found to have pro-apoptotic properties. RNase L is thought to be a mediator of apoptosis as RNaseL -/- mice, and overexpression of RNaseL *in vitro,* has lead to suppression and induction of cell death respectively (Zhou et al., 1997; Clemens, 2003). Also, the 9-2 isozyme of 2',5' OAS has been suggested to interact with anti-apoptotic Bcl-2 family members, thus promoting apoptosis (Ghosh et al., 2001). The OAS/RNaseL pathway could also be pro-apoptotic by degrading transcripts coding anti-apoptotic proteins or cell-survival factors (Barber, 2000).

Virus Induced-Apoptosis in Fish

Viruses have been shown to induce cell death by apoptosis in fish cells. Lymphocystis disease virus (LCDV), an iridoviridae family member, induced apoptosis in the Japanese flounder gill cell line FG-9307 (Hu et al., 2004b). Spring viremia of carp virus (SVCV), a rhabdovirus, killed EPC *via* apoptosis (Bjorklund et al., 1997). ISAV, an orthomyxivirus, was shown to induce apoptosis in the Atlantic salmon head kidney macrophage cell line (SHK-1) and CHSE-124 cells but not in the Atlantic salmon head kidney leukocyte cell line (TO) (Joseph et al., 2004). Also, the rhabodovirus IHNV was shown to induce apoptosis in CHSE-214. It was the IHNV M protein that was found to shut down host transcription and induce apoptosis during viral infection (Chiou et al., 2000).

IPNV is the most studied viral inducer of apoptosis in fish. IPNV, a member of the birnaviridae family, has been shown to induce apoptosis in CHSE-214 cells, prior to necrotic cell death (Hong et al., 1998) and in Atlantic salmon post-smolt hepatocyte primary cultures (Santi et al., 2005). However, the extent of IPNV induced apoptosis in CHSE-214 cultures was later suggested to be a rare event in culture (Espinoza et al., 2005). Mcl-1, a Bcl-2 family member, blocks the action of Bax and inhibits mitochondrial mediated apoptosis. It was found that Mcl-1 levels decreased in IPNV infected CHSE-214 cells, and this decrease was suggested to be mediated by viral proteins (Hong et al., 1999). The IPNV VP5 protein was suggested to play a role in inhibiting host induced apoptosis (Hong and Wu, 2002); however, this role was later questioned, as no anti-apoptotic effect could be detected using IPNV VP5 mutants (Santi et al., 2005). It was also found that IPNV induced apoptosis in zebrafish liver epithelium (ZLE) cell line *via* caspase-8 and -3 induction (Hong et al., 2005). LCDV (Hu et al., 2004b) and ISAV (Joseph et al., 2004) -induced apoptosis was also determined to be caspase dependent. In fact, the ISAV protein, PB2, was found to interact with caspase-8, suggesting a role for this protein in inducing apoptosis (Joseph et al., 2004).

There has been evidence for a role for PKR in virus induced apoptosis. In one study EHNV induced apoptosis in EPC. Treating the cells with 2-aminopurine (2-AP), a PKR inhibitor, reduced EHNV titres 1000-fold, implicating PKR as the mediator of apoptosis (Essbauer and Ahne, 2002). Also, IPNV was shown to induce PKR activity, as measured by eIF2-α phosphorylation, in RTG-2 (Garner et al., 2003). Currently, there has been no investigation into dsRNA-induced apoptosis in fish.

1.2.3. Homotypic aggregation as an antiviral response

Homotypic aggregation (HA) is the adherence of cells of similar cell type. This is an active cell response that requires the up-regulation of adhesion molecules (Fernandes et al., 1999). In leukocytes, the adhesion molecules most commonly associated with HA are lymphocyte function-associated molecule -1 (LFA-1) and intercellular adhesion molecule -1 (ICAM-1) (Weeks and Iuorno, 1996). HA is an important response of immune cells to pathogenic stimuli. It has been observed in many immune cells including: monocytes (Yue et al., 1999), neutrophils (Rochon and Frojmovic, 1992), lymphocytes (Zapata et al., 1995) and eosinophils (Teixeira et al., 1995). HA is important for leukocyte trafficking and activation, cytokine production and antigen presentation (Cho et al., 2003a) as well as immune cell localization to a site of inflammation (Watanabe and Fan, 1998; Neelamegham et al., 2000). HA has been used as a marker of activated immune cells *in vitro*, and has been shown to affect cytokine expression and cellular proliferation (Wake et al., 1995).

Viruses can induce HA and can use this cell adhesion to aid in viral dissemination and pathogenesis (Takahashi et al., 2002). For example, latent Epstein-Barr virus infections in B

lymphocytes trigger HA (Park and Faller, 2002). The human T-cell leukemia virus type I (HTLV-1) Tax protein induces HA in human T cells and it is this aggregation that is believed to aid in the persistence of viral infection and the development of disease (Takahashi et al., 2002). The measles virus (MV) can affect LFA-1 expression levels in human leukocyte cultures, resulting in HA. It has been suggested that this HA may aid in the effective dissemination of the virus between leukocytes and consequently throughout the body (Attibele et al., 1993). Poly IC, a synthetic dsRNA and viral mimic, has also been shown to upregulate adhesion molecule expression, such as E-selectin (Faruqi et al., 1997), vascular cell adhesion molecule (VCAM-1) (Offermann et al., 1995) and ICAM-1 (Yang et al., 1994) in endothelial cells. In fact, poly IC induced VCAM-1 (Offermann et al., 1995) and E-selectin (Bandyopadhyayk et al., 2000) expression was suggested to be mediated *via* PKR, and in the case of VCAM-1 by activating NF-κB (Offermann et al., 1995). Galectins are another family of adhesion molecules involved in immune responses. Galectins are found in epithelium, endothelium and activated tissue macrophages (Almkvist and Karlsson, 2004) and their expression can be up-regulated by poly IC (Ishikawa et al., 2004).

Very little is known of adhesion molecules in fish, especially in regard to leukocyte migration and extravasation (Mulero et al., 2001). Evidence for an integrin fibrinogen receptor has been reported in thrombocytes (Hill and Rowley, 1998) and fibroblasts (Reinhart and Lee, 2002). The VHSV G-protein induced B lymphocyte cell adhesion molecule expression in Japanese flounder juveniles (Byon et al., 2005). HA has been reported in Channel catfish (*Ictalurus punctatus* Rafinesque) neutrophils stimulated with phorbol dibutyrate (PBDU) (Ainsworth et al., 1996). This aggregation was suggested to be mediated by integrins as it was divalent cation-dependent and could be blocked with the addition of the peptide RGD (Arg-Gly-Asp), an integrin binding sequence. Galectins have also been identified in many teleost fish (Vasta et al., 2004) including rainbow trout (*O. mykiss*) (Inagawa et al., 2001). Interestingly, expression of a galectin-9-like molecule was detected in VHSV exposed rainbow trout leukocytes (O'Farrell et al., 2002).

1.3. CELL LINES AND STUDYING ANTIVIRAL MECHANISMS

Cell lines play an important role in the study of animal viruses and aid in the understanding of host antiviral mechanisms. There are at least five major areas where cell lines aid in the understanding of viruses themselves. Firstly, cell lines are needed to produce sufficient virus to characterize the biophysical and biochemical properties of a virus. Examples of such characterization would include describing viral proteins and genome organization (Winton et al., 1987). Secondly, cell cultures are needed to study the single-cell reproductive cycle of virus. This would include understanding the molecular events involved in the entry, replication, assembly and

release of viruses (Rivas et al., 1998; Duncan, 1996). Thirdly, cell lines can contribute to preventing and controlling viral diseases by being a source of viruses for vaccines and an experimental system for the development of antiviral drugs (Corbeil et al., 2000). Fourthly, in some cases, cell lines can advance an understanding of viral pathogenesis. For example, CHSE-214 was used as a tool for studying the characteristics of IHNV persistent infections found both in the cell lines and in whole fish (Engelking and Leong, 1981). Finally, cell lines are powerful diagnostic tools, for discovering new viruses and identifying viral pathogens from environmental samples (Amend, 1975).

The role cell lines have played in studying host antiviral mechanisms has been invaluable,as they have allowed for a detailed study of host responses during every step of viral infection while offering reproducibility and cell type specificity. In fish, cell lines have aided in at least four important aspects of understanding antiviral mechanisms. Firstly, cell lines have been invaluable for studying antiviral gene expression (Nygaard et al., 2000) and gene promoter studies (Collet et al., 2004; Johansen et al., 2004). More specifically, cell lines have enabled studies of the signaling pathways involved, such as identifying and elucidating interferon signaling mechanisms (Zhang and Gui, 2004; Collet and Secombes, 2002). Secondly, cell lines have been important for identifying and characterizing new antiviral proteins, such as the PKR-like gene (Hu et al., 2004a), Mx (Trobridge and Leong, 1995; Trobridge et al., 1997b), IRF-1 and IRF-2 (Collet et al., 2003), and eIF2α (Garner et al., 2003). Thirdly, cell lines are useful for generating recombinant proteins, such as interferon, and for studying the effects of these proteins at the cellular level (Long et al., 2004). Fourthly, cell lines have allowed for the study of virus-induced cell death and its mechanisms, specifically by apoptosis (Bjorklund et al., 1997; Hong et al., 1998; Hu et al., 2004b).

1.3.1. Fish monocyte/macrophage cell lines

A benefit of cell lines is cell type specificity, which allows researchers the ability to study the responses of one cell type in a controlled environment. RTS11, a rainbow trout macrophage-like cell line, has proven to be a valuable tool for studying immune cell –specific responses *in vitro*. RTS11 was initiated from a long-term haemaopoietic cell culture of a rainbow trout spleen (Ganassin and Bols, 1998). RTS11 secrete lysozyme, are able to phagocytose (Ganassin and Bols, 1998) and express major histocompatibility complex (MHC) class II β at the transcript level (Brubacher et al., 2000). RTS11 is also able to respond to lipopolysaccharide by expressing interleukin (IL)-1 β, tumour necrosis factor (TNF)-α and cyclooxygenase (cox)-2 (Brubacher et al., 2000). RTS11 have not as of yet been shown to mount a respiratory burst in response to a variety of stimuli.

Other teleost macrophage-like cell lines have also been developed. CLC (carp peripheral blood leukocyte) was the first macrophage-like cell line described in fish, and was initiated from the peripheral blood of the common carp (Faisal and Ahne, 1990). CLC is able to mount a respiratory

burst in response to LPS and phorbol 12-myristate 13-acetate (PMA) (Weyts et al., 1997). Peripheral blood leukocytes of channel catfish have been the source of several monocyte-like cell lines (Vallejo et al., 1991). GMLC, another fish macrophage cell line, was developed from goldfish kidney, and was able to mount a respiratory burst in response to LPS (Wang and Belosevic, 1995). Finally, there has been a macrophage-like cell line derived from Atlantic salmon, SHK-1. SHK-1 reacted to monoclonal antibodies against Atlantic salmon peripheral blood leukocytes (Dannevig et al., 1997) and were able to phagocytose bacteria (Koppang et al., 1999).

1.4. GOALS OR AIMS OF THIS WORK

The goal of this thesis was to use cell lines as a tool to better define antiviral mechanisms in fish. Four cell lines were used in this study, RTS11, a rainbow trout macrophage-like cell line, RTG-2, a rainbow trout gonadal fibroblast cell line, CHSE-214, a Chinook salmon embryonic cell line, and PBLE, a new continuous cell line derived from a peripheral blood leukocyte preparation of the American eel, *Anguilla rostrata*. Both synthetic dsRNA and live viruses are used as stimuli for studying three different antiviral responses: apoptotic cell death, antiviral gene expression and homotypic aggregation.

Firstly, poly IC-induced apoptosis was investigated in RTS11, RTG-2 and CHSE-214. Secondly, this dsRNA-induced apoptosis was compared with apoptotic cell death induced by gliotoxin, a fungal secondary metabolite, and differences were found between cell lines and between stimuli. Thirdly, induction pathways of two antiviral genes, *Mx* and *vig-1*, were studied in RTS11 and RTG-2 following exposures with dsRNA, interferon, and chum salmon reovirus (CSV). Fourthly, homotypic aggregation was described in RTS11 following treatments with dsRNA and CSV. Cell responses to CSV were investigated in all four cell lines, including cytopathic effect (CPE) and virus-induced cell death. Finally, PBLE was described for the first time, and its responses to gliotoxin and CSV were examined.

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Chapter 2

Preferential induction of apoptosis in the rainbow trout macrophage cell line, RTS11, by actinomycin D, cycloheximide and double stranded RNA

ABSTRACT

The rainbow trout macrophage cell line RTS11 was found to be considerably more sensitive than rainbow trout fibroblast (RTG-2) and Chinook salmon epithelial (CHSE-214) cell lines to killing by macromolecular synthesis inhibitors, actinomycin D (AMD) and cycloheximide (CHX), a synthetic double stranded RNA (dsRNA), polyinosinic: polycytidylic acid (poly IC), and combinations of poly IC with AMD or CHX. Exposures of 24 - 30 h to AMD or CHX alone killed RTS11, but not CHSE-214 and RTG-2, in basal medium, L-15, with or without fetal bovine serum (FBS) supplementation. A two-week exposure to poly IC killed RTS11 in L-15, whereas RTG-2 and CHSE-214 remained viable. At concentrations that caused very little or no cell death, CHX or AMD pretreatments or co-treatments sensitized RTS11 to poly IC, causing death within 30 h. In all cases death was by apoptosis as judged by two criteria. H33258 staining revealed a fragmented nuclear morphology, and genomic degradation into oligonucleosomal fragments was seen with agarose gel electrophoresis. With AMD- or CHX-induced death, killing seemed caspase-independent as the pan caspase inhibitor, zVAD-fmk, failed to block killing. By contrast, zVAD-fmk almost completely abrogated killing by co-treatments of poly IC and low concentrations of AMD or CHX, suggesting caspase dependence. Killing by both types of treatments was blocked by 2 aminopurine (2-AP), which suggests the involvement of dsRNA-dependent protein kinase (PKR). The sensitizing of RTS11 to poly IC killing by AMD or CHX could be explained by a decrease in the level of a short-lived anti-apoptotic protein(s) and/or by the triggering of a ribotoxic stress.

2.1. INTRODUCTION

Synthetic double-stranded RNAs (dsRNA), such as polyinosinic: polycytidylic acid (poly IC), have been valuable agents for dissecting the antiviral mechanisms of mammalian cells. Although normally the cellular levels of dsRNA are extremely low, they increase profoundly during virus infections, as most viruses produce dsRNA at some point during their reproductive cycle (Jacobs and Langland, 1996). Exposing cells in culture to poly IC has been used for over 30 years as a simple, convenient way of studying the responses of mammalian cells to dsRNA. Some of the first documented responses in mammalian cells to poly IC were the inhibition of cell proliferation, induction of interferons (IFNs), and cell death (Stewart et al., 1972).

The killing of mammalian cells by dsRNA varies with cell type and context of dsRNA exposure (Kalai et al., 2002; Yeung et al., 1996; Der et al., 1997; Takizawa et al., 1996; Srivastava et al., 1998; Lee and Esteban, 1993; Lee and Esteban, 1994), and appears to be part of an antiviral mechanism, serving to limit the spread of viral infection (Everett and McFadden, 1999). In most cases, the death induced by poly IC is apoptosis or programmed cell death and is mediated by dsRNA-dependent protein kinase (PKR)(Gil and Esteban, 2000a). PKR appears to function through the FADD (Fas-associated death domain)/caspase 8 pathway (Gil and Esteban, 2000b; Balachandran et al., 1998). FADD functions primarily as a death receptor adaptor molecule essential for the recruitment and activation of specific procaspases, such as caspase-8. Procaspases are zymogens that are activated through proteolysis and lead to apoptosis. A feature of the FADD/caspase 8 pathway is sensitization by transcriptional and translational inhibitors, such as actinomycin D and cycloheximide (Fulda et al., 2000).

Piscine cell lines and dsRNA have been used to study antiviral mechanisms in fish. Initially poly IC was shown to induce protection to IPNV in RTG-2, a rainbow trout gonadal fibroblast cell line, but not in CHSE-214, a Chinook salmon embryonic cell line (MacDonald and Kennedy, 1979). Subsequently, poly IC treatment protected different cell lines from infection by IPNV (infectious pancreatic necrosis virus) (Jensen et al., 2002b), ISAV (infectious salmon anaemia virus) (Jensen et al., 2002a; Jensen and Robertsen, 2002) and SHRV (snakehead rhabdovirus) (Altmann et al., 2003). This protection suggests the induction of interferon. With the recent identification of teleost type I interferon, direct evidence of interferon induction by poly IC has been obtained with cell lines from the channel catfish (CCO) and zebrafish (ZFL) (Altmann et al., 2003; Long et al., 2004). More commonly, the expression of interferon-inducible genes, including Mx and transcription factors IRF-1 and IRF-2, whose induction can be taken as indirect evidence of interferon production, has been studied. Poly IC-induced expression of Mx, IRF-1 and IRF-2

has been studied in RTG-2 (Trobridge and Leong, 1995; Trobridge et al., 1997b; Collet and Secombes, 2001; Collet and Secombes, 2002; Collet et al., 2003). Mx induction has also been observed in CHSE-214 (Trobridge and Leong, 1995; Jensen et al., 2002b; Nygaard et al., 2000), and several Atlantic salmon cell lines (Jensen and Robertsen, 2002; Nygaard et al., 2000). In the rainbow trout monocyte/macrophage cell line, RTS11, poly IC enhanced the expression of interferon-inducible chemokines (Laing et al., 2002a; Laing et al., 2002b). The phosphorylation of eIF2α in RTG-2 and ZFL was stimulated by poly IC, suggesting the presence of a PKR (Garner et al., 2003). Despite these advances, the cytotoxic effects of dsRNA have yet to be investigated in teleost cell lines, although viral-induced apoptosis with the involvement of PKR has been described recently (Essbauer and Ahne, 2002; Chinchar et al., 2003).

Understanding apoptosis in fish could contribute to understanding diseases in general and perhaps viral diseases in particular. Therefore, we have studied the capacity of dsRNA alone or together with inhibitors of macromolecular synthesis to kill the salmonid cell lines, RTS11, RTG-2 and CHSE-214. Cell viability was measured with two fluorescent dyes, alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), which monitor metabolism and cell membrane integrity respectively (Dayeh et al., 2003). Apoptosis was investigated by looking for characteristic nuclear changes and by determining the effect of the pan-caspase inhibitor zVAD-fmk, on cell viability. The nuclear changes monitored were the appearance of DNA ladders upon gel electrophoresis of genomic DNA, nuclear fragmentation as viewed by fluorescent microscopy of H33258 stained cells, and in some cases chromatin condensation as revealed by transmission electron microscopy (TEM). RTS11 is of particular interest because macrophages play a central role in virus-host interactions (Humlova et al., 2002). The work represented in this chapter has been published (DeWitte-Orr et al., 2005).

2.2. MATERIALS AND METHODS

2.2.1. Materials

Sigma (St Louis, MO) was the source of polyinosinic-polycytidylic acid (poly IC), polyinosinic acid (poly I), polycytidylic acid (poly C), cycloheximide, actinomycin D, 2-aminopurine, GenElute™ mammalian genomic DNA miniprep kit, Leibovitz's L-15, penicillin G, streptomycin sulfate, Dulbecco's phosphate buffered saline (D-PBS), and fetal bovine serum (FBS). Riedel-de Haen Ag Seetz-Hannover was the source for Hoechst 33258 dye. The caspase inhibitor, zVAD-fmk, was purchased from Calbiochem (La Jolla, CA). Plastic tissue culture flasks and slide flasks were manufactured by Nunc (Roskilde, Denmark); 96 well plates, by Falcon/Becton-Dickenson (Franklin Lakes, NJ). For incorporation studies, 24 well PET plates, Optiphase Supermix Scintillation fluid and MicroBeta reader from Wallac (Turku, Finland) were used and cells were labeled with either L- [4,5-^{3}H] leucine (164 Cimmol^{-1}) or [5,6-^{3}H] uridine (41.0 Cimmol^{-1}) from Amersham Biosciences (UK).

2.2.2. Cell Culture

Three cell lines were used in the course of this study: fibroblast-like RTG-2, monocyte/macrophage RTS11 and epithelial-like CHSE-214. RTG-2 and CHSE-214 were obtained from the American Type Culture Collection (ATCC), while RTS11 was developed in this laboratory (Ganassin and Bols, 1998). Both RTG2 and CHSE-214 cells were cultured at 21^{o}C in 75 cm^{2} tissue culture treated flasks, in L-15 medium supplemented with 150 U mL^{-1} penicillin G, 150 μg mL^{-1} streptomycin sulfate, and 10% fetal bovine serum (FBS). RTS11 cells were grown at 18^{o}C in 25cm^{2} tissue culture treated flasks in the same medium but with 15% FBS. RTG-2 and CHSE-214 grow as strictly adherent cells, whereas growing RTS11 cultures have the majority of cells in suspension or loosely adherent but a minority are adherent. Confluent RTG2 and CHSE-214 flasks were passaged every two weeks by splitting them 1:4. RTS11 were passaged every three to four weeks, the culture and conditioned medium was split into two flasks, and an equal volume of fresh medium was added to each flask. The passage numbers for RTS11 used in this study were 30-58.

2.2.3. Cytotoxicity Assay

Plating and dosing

Cells were seeded into 96 well plates, in L-15 growing medium with or without the FBS supplement. CHSE-214 cells were seeded at 3.0 x 10^{4} cells per well, RTG2 at 5.0 x 10^{4} cells per well, and RTS11 at 1.25 x 10^{5} cells per well. Cells were allowed to settle and reattach for 24 h at

their normal growing temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of poly IC, poly I, poly C, cycloheximide, actinomycin D, 2-aminopurine or zVAD-fmk. For potentially toxic solvents (such as DMSO or ethanol), other cells were treated with an equal volume of the carrier itself as a control. After a specific incubation time, cultures were evaluated for cytotoxicity. In no case was the solvent used at a concentration that was cytotoxic. In most cases, results were calculated as a percent of the carrier-treated control cells.

Measuring cell viability

Two fluorescent indicator dyes, alamar Blue™ (Medicorp, Montreal, PQ) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Molecular Probes, Eugene, OR) were used to evaluate cell viability. Detailed protocols on the use of these dyes and the interpretation of the results have been described previously (Dayeh et al., 2003).

2.2.4. Determining Apoptosis

For evidence of apoptosis, cultures were evaluated for nuclear fragmentation as assessed by Hoechst staining and genomic DNA fragmentation. Unless otherwise indicated, RTS11 cells were seeded at $2.0x10^6$ cells in a $9cm^2$ slide flask and $7.0x10^6$ cells in a $12.5cm^2$ tissue culture treated flask respectively. These cells were seeded in L-15 growing medium, and incubated at 18^oC for 24 h. The cells were then dosed with specific combinations of poly IC, cycloheximide, and actinomycin D. The cells were then incubated at 18^oC for the required amount of time. Poly IC stimulated apoptosis after 14 days in RTS11 were determined by TEM analysis as well.

Hoechst 33258 stain

After the indicated exposure time, RTS11 cultures were fixed by adding an equal volume of Carnoy's fixative (methanol: glacial acetic acid, 3:1), which was prepared fresh with each use, to existing media, exposing the cells for 2 min. Following the initial fixation, the media and fixative was removed and the cells were exposed to fresh fixative twice, each incubation being 5 min. The slides were air-dried and the cells were then stained with $0.5\mu g\ mL^{-1}$ Hoechst (H) 33258 for 10 min., followed by a rinse in deionized water. After drying, a coverslip was mounted onto the slide in PBS with glycerol (1:1). The fluorescent nuclei were visualized using a fluorescent microscope with a ultra-violet (UV) filter (Nikon Optishot). A total of 300 nuclei in three randomly chosen fields on each slide were scored as having the characteristic fragmented appearance of apoptotic cells or not.

DNA fragmentation ladder

After a specified treatment, cells were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit (Sigma), which is based on column affinity technology. The genomic DNA was eluted from the column using 100 µL Milli-Q water, and 35 µL of this DNA was resolved by electrophoresis on a 2% (w/v) agarose gel for 4 h at 60V. The DNA ladders were visualized by staining gels with 0.5 µg mL^{-1} ethidium bromide and photographed under UV transillumination.

Transmission Electron Microscopy

RTS11 were seeded in $25cm^2$ tissue culture treated flasks at $1.05x10^7$ cells per flask in L-15 growing medium. After 24 h incubation at 18°C to allow reattachment, the cells were dosed with 50 µg mL^{-1} poly IC, and incubated again at 18°C for ten days. When the poly IC treated cells began to show signs of dying they were collected and pelleted using a swinging bucket centrifuge. The cell pellet was resuspended in 0.2 M phosphate buffer (pH 7.2) for 30 min. The phosphate buffer was then replaced and cells were incubated overnight in a 0.2M phosphate buffer with 2.5% gluteraldehyde (pH 7.2). The cells were then washed three times with 0.2 M phosphate buffer (pH 7.2) and incubated in 1% OsO_4 solution for 2 h. The OsO_4 solution was then removed by washing the cells twice in distilled water for 30 min. The cells were then dehydrated in a series of graded acetone (to 100%). The dehydrated cells were embedded in Epon-Araldite, in Beem capsules. Sections of the embedded cells were taken, at 60-90nm, using an ultramicrotome (Reichert Ultracut E). These sections were placed on 3mm copper grids and stained with freshly prepared lead citrate for 10 min., after which they were rinsed with distilled water. The cells were then stained with freshly prepared saturated uranyl acetate stain in 70% ethanol. To remove the stain, the sections were washed in 70% ethanol and dried. Prepared sections were viewed using a transmission electron microscope (Philips CM10).

2.2.5. Metabolic labeling studies

RTS11 were seeded in 24 well PET plates with normal growth medium and allowed to attach overnight at 18°C. For measuring protein synthesis, cells were washed in leucine-reduced media and incubated for 2h with or without CHX treatment (0.1 and 10 µg mL^{-1}). Cultures were then labeled with L- [4, 5-^{3}H] leucine for another 2hr. RNA synthesis of RTS11 with or without AMD treatment (0.5 and 5 µg mL^{-1}) was monitored by measuring [5, 6-^{3}H] uridine incorporation in serum free L-15. Macromolecules were precipitated with 10% trichloroacetic acid and washed two times in cold D-PBS. Optiphase Supermix scintillation fluid was added to the wells, and

incorporation of radiolabelled probes was quantified using scintillation counting (MicroBeta, Wallac).

2.2.6. Data analysis

For alamar Blue and CFDA-AM assays, cytotoxicity was indicated by a decline in fluorescence units (FUs) for experimental cultures relative to control cultures. FUs for culture wells with no cells were constant and subtracted from FUs for experimental and control cultures. In some cases, where different treatments were being compared, such as in Figure 2-3, FUs between cultures were analyzed by a one-way ANOVA, and when warranted ($p \leq 0.05$), followed by Tukey-Kramer post test. For graphical presentation, the results were plotted using SigmaPlot (Jandel Scientific), and EC_{50} values were determined using the logistic function option in SigmaPlot for dose-response curves (Dayeh et al., 2003). EC_{50} values for the different assays were compared by an unpaired t-test. For the number of apoptotic nuclei in cultures after different experimental treatments, a one-way ANOVA with Tukey-Kramer post test was performed ($p \leq 0.05$). All statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com).

2.3. RESULTS

2.3.1. CHX and AMD induced apoptosis in RTS11

The macromolecular synthesis inhibitors, cycloheximide (CHX) and actinomycin D (AMD) killed RTS11 in a dose-dependent manner after 24h and 30h treatments respectively. This occurred whether RTS11 were in L-15 alone (Figure 2-1A and B) or in L-15 with FBS (data not shown). Under the same exposure conditions, neither CHX nor AMD treatment caused cytotoxicity in RTG-2 and CHSE-214 cultures (Figure 2-1C-F), although exposures of 48h or more ultimately did kill these cell lines (data not shown). In L-15 alone the EC_{50} values for killing by CHX and AMD were respectively 0.70 $\mu g\ ml^{-1}$ ± 0.64 (n=5) and 1.43 $\mu g\ mL^{-1}$ ± 1.25 (n=3) as evaluated by alamar Blue and 0.54 ± $\mu g\ mL^{-1}$ 0.23 (n=3) and 1.61 $\mu g\ mL^{-1}$ ± 1.22 (n=3) as evaluated by CFDA-AM. At concentrations that killed RTS11, CHX and AMD induced DNA ladders (Figure 2-2A) and nuclear fragmentation (Figure 2-2B-D), which suggests apoptosis. The percentage of fragmented nuclei in control, CHX (10 $\mu g\ mL^{-1}$) and AMD (5 $\mu g\ mL^{-1}$) treated cultures were 2.33 ± 2.08, 91.3 ± 9.50, and 74.67 ± 3.21 (n=3) respectively, with the values for treated cultures being significantly different from the control ($p<0.001$). Cultures treated with the macromolecular inhibitors plus zVAD-fmk continued to show fragmented nuclei and gave FU values for both alamar Blue and CFDA-AM that were not statistically different from cultures treated with the macromolecular inhibitors alone (Figure 2-3). CHX- and AMD- induced killing was totally blocked by 1.5 mM 2-AP, which suggests the involvement of PKR (Table 2-1). Treating RTS11 cultures for 2 h with CHX at 10 $\mu g\ mL^{-1}$ or AMD at 5 $\mu g\ mL^{-1}$ inhibited ^{3}H-leucine and ^{3}H-uridine incorporation by 94.80% ± 4.98 (n=2) and 98.49% ± 0.40 (n=2) respectively. A relationship between the extent of cytotoxicity and the magnitude of macromolecular inhibition was not obvious. Concentrations of CHX (0.1$\mu g\ mL^{-1}$) and AMD (0.5 $\mu g\ mL^{-1}$) that caused slight or no cytotoxicity also inhibited ^{3}H-leucine and ^{3}H-uridine incorporation by 84.81 % ± 5.70 (n=2) and 93.56% ± 1.44 (n=2) respectively. CHX and AMD continued to inhibit precursor incorporation in the presence of 1.5 mM 2-AP. This suggests that inhibiting macromolecular synthesis is a trigger that requires further signaling for the induction of apoptotic death of RTS11.

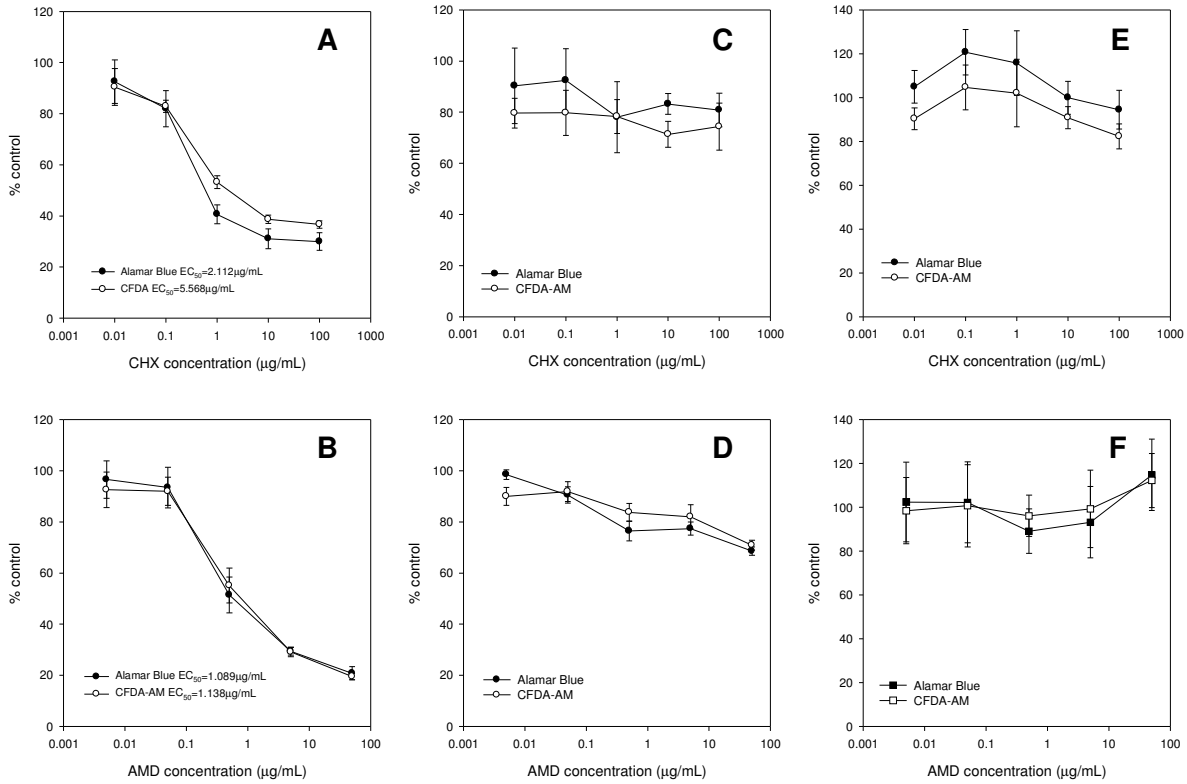


Figure 2-1. A survey of the cytotoxicity of macromolecular synthesis inhibitors, cycloheximide and actinomycin D, on three teleost cell lines.

Cultures of each cell line were exposed to cycloheximide (CHX) or actinomycin D (AMD) for 24 or 30 hrs respectively in L-15 media without FBS. Cytotoxicity was measured using two fluorescent indicator dyes, alamar Blue and CFDA-AM, and was expressed on the Y-axis as a % of control cultures treated with the carrier alone. The x-axis represents the concentration of inhibitor in µg mL^{-1} on a logarithmic scale. RTS11 die in response to CHX (**A**) and AMD (**B**) in a dose dependent manner. No cell death was detected in CHX (**C**) and AMD (**D**) treated RTG-2 or CHX (**E**) and AMD (**F**) treated CHSE-214.

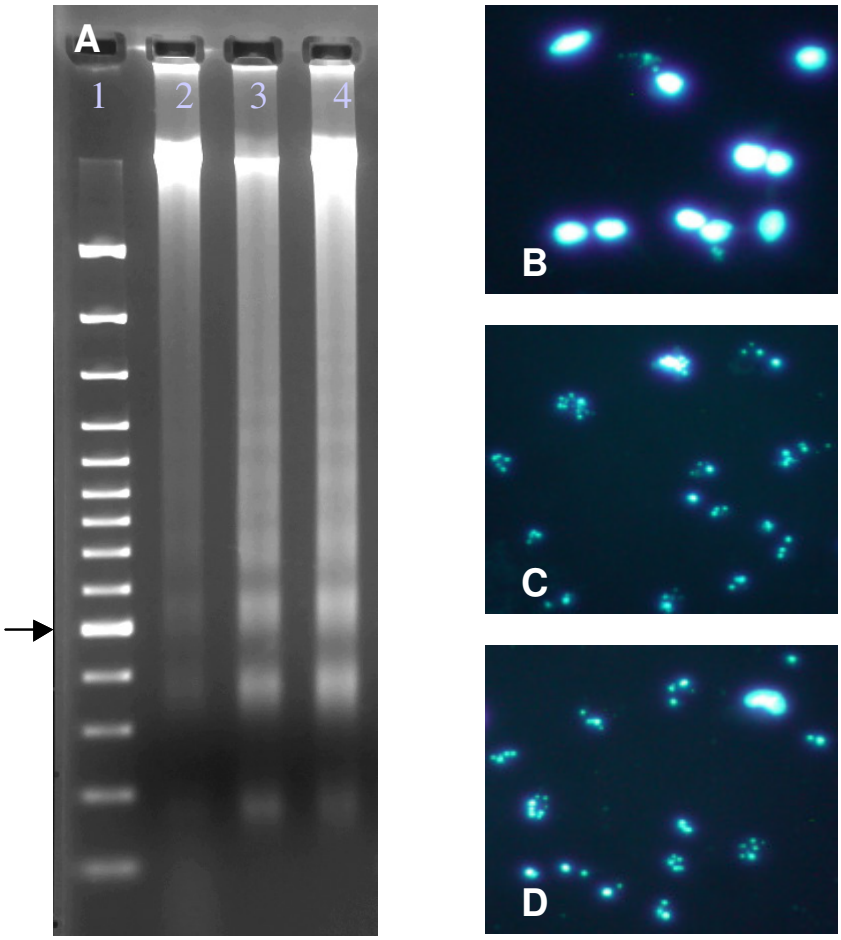
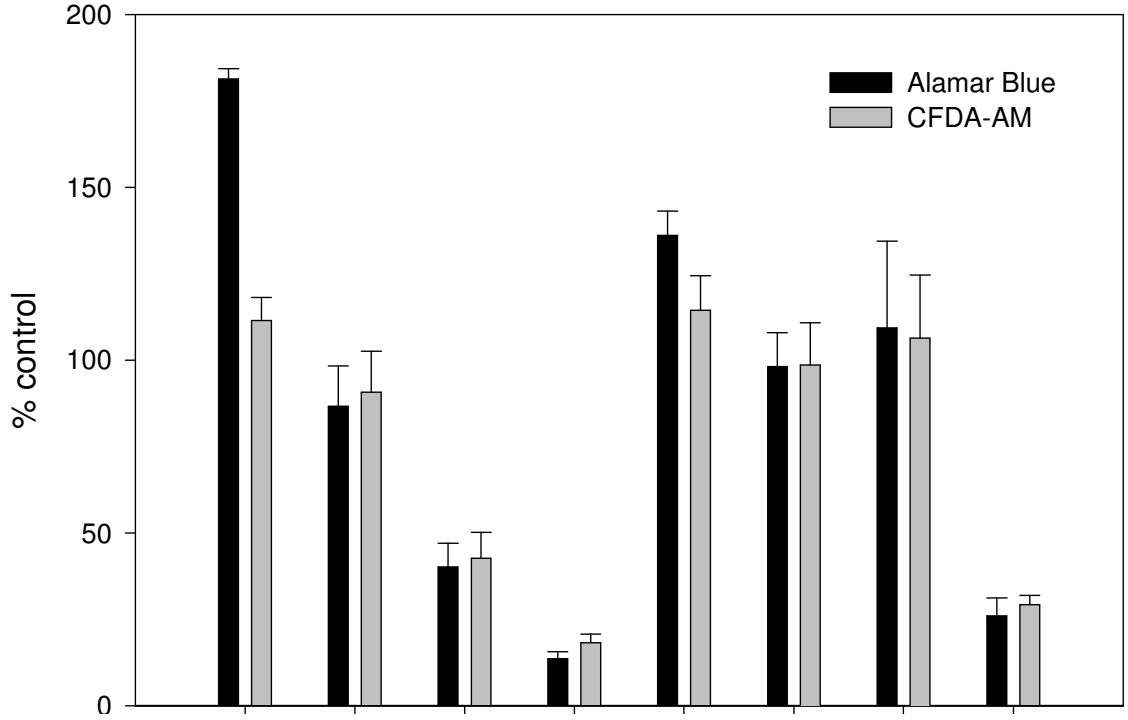


Figure 2-2. Macromolecular synthesis inhibitors, cycloheximide and actinomycin D, kill RTS11 by apoptosis.

Cultures in L-15 were exposed to either cycloheximide (CHX) for 24 h or actinomycin D (AMD) for 30 h and then analyzed for hallmarks of apoptosis. In panel **A** genomic DNA (gDNA) was extracted from control (lane 2), 10 µg ml^{-1} CHX-treated (lane 3), and 5 µg ml^{-1} AMD-treated (lane 4) cultures, run on 2 % agarose gel, and stained with EtBr. Lane 1 is the 100 bp DNA marker with the arrow identifying 500 bp. The remaining panels reveal by Hoechst 33258 staining and fluorescence microscopy the nuclear morphologies in control cultures (**B**), and in cultures treated with (**C**) 10 µg ml^{-1} CHX or (**D**) 5 µg ml^{-1} AMD. All pictures were taken at 400X magnification.



PolyIC (µg/mL)	50		50		50		50	
AMD (µg/mL)		0.5	0.5	50		0.5	0.5	50
zVAD-fmk (µM)					50	50	50	50

Figure 2-3. Effect of a caspase inhibitor on the cytotoxicity of poly IC, AMD, and co-treatments of the two in RTS11.

Cultures were pretreated with 50 µM zVAD-fmk, a pan-caspase inhibitor, for 1 hour prior to treatment with poly IC and AMD for 30 h. The Y-axis represents cell viability, which was evaluated with alamar Blue and CFDA-AM, and expressed as a % of the control (cells treated with the carrier alone), while the values under the X-axis refer to specific combinations of experimental treatments. Co-treatments with poly IC and CHX yielded similar results (data not shown).

2.3.2. Poly IC together with either CHX or AMD induced apoptosis in RTS11

Poly IC, together with a concentration of CHX or AMD that alone caused slight or no cytotoxicity, killed RTS11 in a dose-dependent manner after 24 to 30 h exposures in L-15 alone (Figure 2-4A and B). This killing was blocked by 1.5 mM 2-AP (Figure 2-5 and Table 2-1). EC_{50} values for killing by poly IC with 0.1 μg mL^{-1} CHX were 31.72 μg mL^{-1} ± 4.61 (n=3) and 24.66 μg mL^{-1} ± 3.76 (n=3) as evaluated respectively by alamar Blue and by CFDA-AM. EC_{50} values for killing by poly IC with 0.5 μg mL^{-1} AMD were 10.86 μg mL^{-1} ± 7.00 (n=3) and 11.60 μg mL^{-1} ± 5.54 (n=3) as evaluated respectively by alamar Blue and by CFDA-AM. By contrast, no combination of poly IC and CHX or AMD caused a loss of cell viability in RTG-2 or CHSE-214 cultures under the same exposure conditions (data not shown). In medium with or without FBS, poly IC alone at up to 500 μg mL^{-1} for 30 h failed to kill any of the cell lines. In contrast to death by high concentrations of CHX or AMD alone, RTS11 did not die when exposed to the co-treatments of poly IC and either CHX or AMD in L-15 with FBS.

Poly IC together with either CHX or AMD appeared to kill RTS11 by apoptosis. Genomic DNA run on an agarose gel demonstrated the distinctive laddering pattern of 180 bp oligomers, indicating internucleosomal degradation (Figure 2-4C). Nuclear fragmentation was clear from H33258 staining of treated RTS11 (Figure 2-4D-G). In control cultures and cultures with poly IC (50 μg ml^{-1}) alone, the numbers of fragmented nuclei were less than 10 % and not statistically different from one another, whereas the % of apoptotic nuclei in cultures with CHX (0.1 μg ml^{-1}) or AMD (0.5 μg ml^{-1}) alone were much higher at 37.3 % ± 4.16(n=3) and 38 % ± 4.00 (n=3) respectively. Cultures with poly IC together with either one of the macromolecular inhibitors had over 70% of the nuclei being apoptotic and these numbers were statistically different from all other cultures ($p<0.001$). Unlike the killing caused by CHX or AMD alone, the pan-caspase inhibitor, zVAD-fmk, inhibited the loss of viability induced by poly IC together with CHX or AMD (Figure 2-3). For cultures with poly IC and macromolecular inhibitors, the addition of zVAD-fmk significantly elevated the FU values from both the alamar Blue and CFDA-AM assays ($p<0.001$).

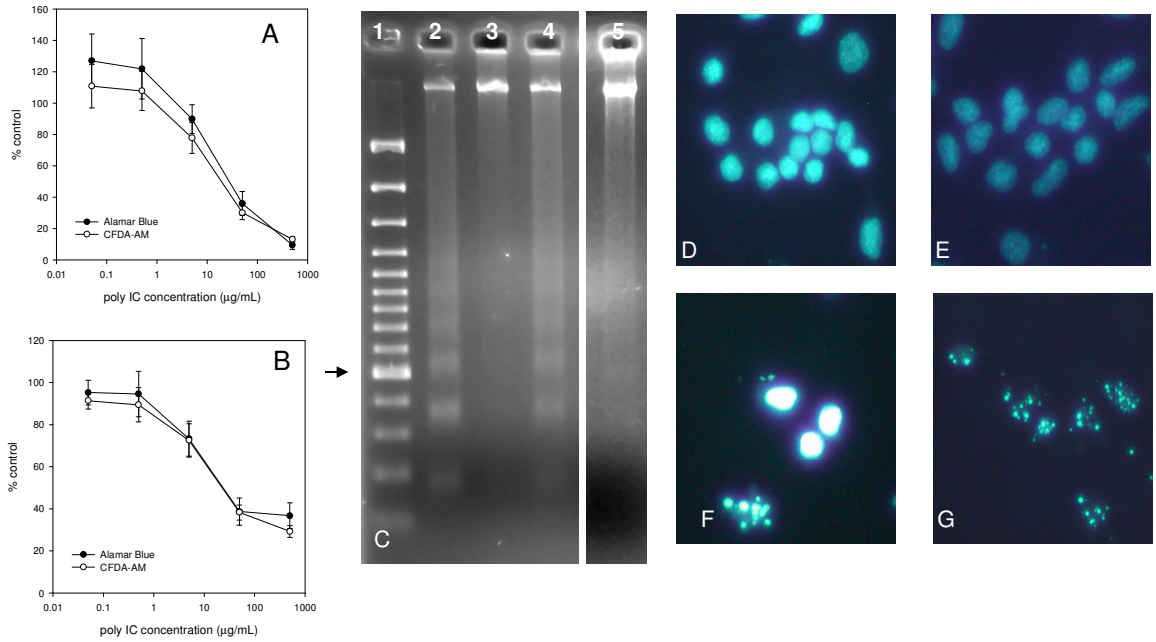


Figure 2-4. Cycloheximide and actinomycin D reduce the exposure time necessary for poly IC to induce apoptosis in RTS11.

Cultures in L-15 were exposed to poly IC and non-toxic concentrations of CHX or AMD for 24h or 30h respectively. In panels **A** and **B**, the Y-axis represents cell viability, which was evaluated with alamar Blue and CFDA-AM, and expressed as a % of the control, with the control being cells exposed to the inhibitors alone. The X-axis shows increasing concentrations of poly IC (µg mL^{-1}) on a logarithmic scale. In panel **A** all cultures were treated with 0.1 µg mL^{-1} CHX, whereas in **B** all cultures were exposed to 0.5 µg mL^{-1} AMD, in combination with increasing concentrations of poly IC. In panel **C** gDNA was run on a 2 % agarose gel and stained with EtBr. Lane 1 is a 100 bp DNA ladder with an arrow identifying 500 bp. The other lanes contain gDNA extracted from cultures exposed to 0.1 µg mL^{-1} CHX with 50 µg mL^{-1} poly IC (lane 2), 50 µg mL^{-1} poly IC (lane 3), 0.5 µg mL^{-1} AMD with 50 µg mL^{-1} poly IC (lane 4) and untreated RTS11 (lane 5). The remaining panels show nuclear morphologies in control cultures (**D**) and in cultures treated with (**E**) 50 µg mL^{-1} poly IC, (**F**), 0.1 µg mL^{-1} CHX alone and (**G**) 0.1 µg mL^{-1} CHX with 50 µg mL^{-1} poly IC as revealed by Hoechst 33258 staining and fluorescence microscopy. All pictures were taken at 400X magnification.

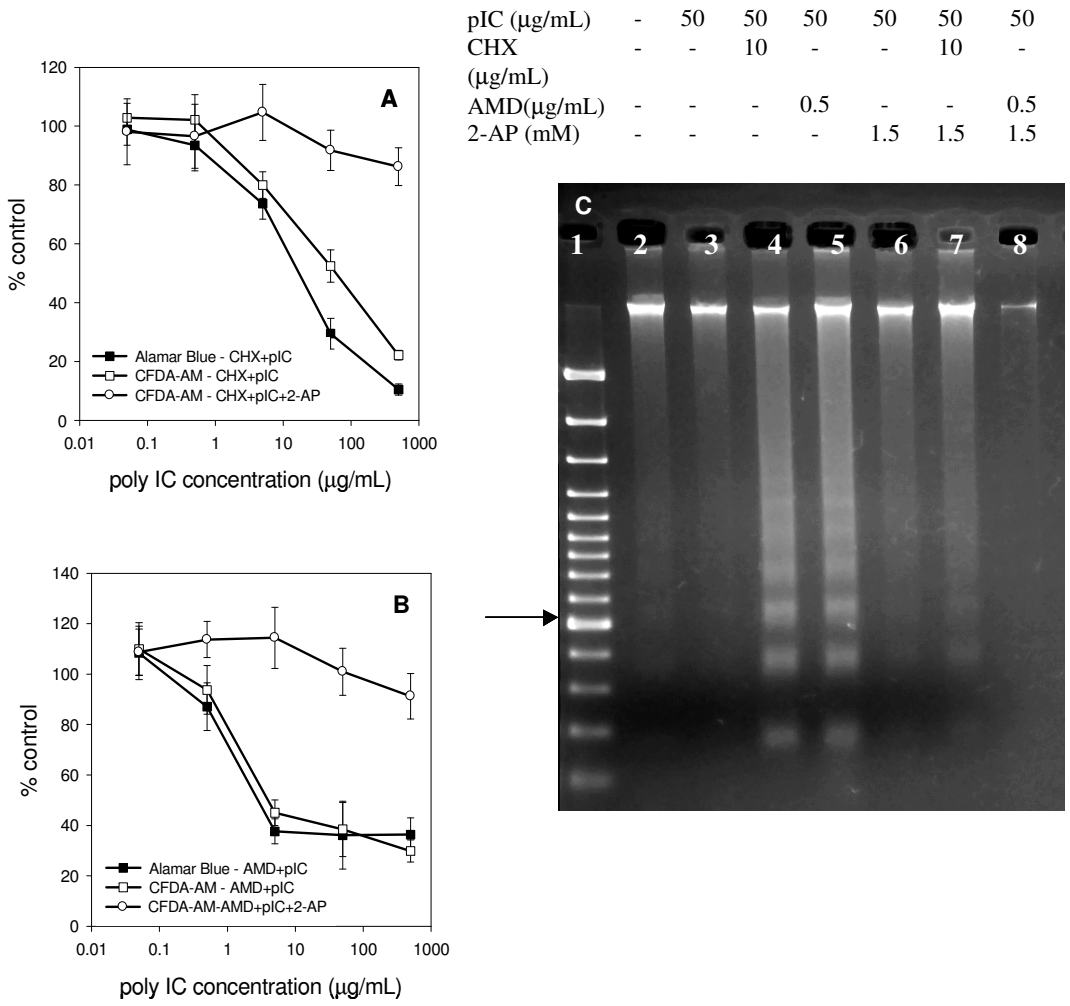


Figure 2-5. Effect of 2-aminopurine on the killing of RTS11 by combinations of poly IC and macromolecular synthesis inhibitors.

Cultures were treated with or without 1.5 mM 2-aminopurine (2-AP) and varying concentrations of poly IC with a fixed concentration of either 10 µg mL^{-1} CHX for 24 h (**A**) or 0.5 µg mL^{-1} AMD for 30 h (**B**). In panels A and B, the Y-axis represents cell viability, which was evaluated with alamar Blue and CFDA-AM, and expressed as a % of the control, which are cells treated with the inhibitor, or the inhibitor and 2-AP.The X-axis shows increasing concentrations of poly IC. In panel **C** DNA was run on a 2 % agarose gel and stained with EtBr. Lane 1 is a 100 bp DNA ladder with an arrow identifying 500 bp. The other lanes contain gDNA extracted from a control culture (lane 2) and from cultures treated with various combinations of poly IC (pIC), CHX, AMD and 2-AP, lanes 3 to 8.

Table 2-1. Effect of 2-aminopurine on the cytotoxicity of CHX, AMD and poly IC alone or in combination to RTS11[1].

Treatment	+ 2-AP (mM)	alamar Blue % cell viability (stdev)[2]	CFDA-AM % cell viability (stdev)[2]
50 μgmL^{-1} poly IC		140.14 (23.44)	83.81 (5.30)
10 μgmL^{-1} CHX		31.07 (3.85)	38.69 (1.64)
10 μgmL^{-1} CHX	1.5	103.56 (13.88)	87.20 (3.45)
0.1 μgmL^{-1} CHX		78.85 (2.25)	94.03 (2.37)
0.1 μgmL^{-1} CHX + 50 μgmL^{-1} poly IC		26.64 (4.68)	44.59 (7.98)
0.1 μgmL^{-1} CHX + 50 μgmL^{-1} poly IC	1.5	93.99 (3.42)	101.47 (3.51)
5 μgmL^{-1} AMD		37.48 (4.26)	58.02 (6.38)
5 μgmL^{-1} AMD	1.5	96.89 (8.76)	98.73 (11.46)
0.5 μgmL^{-1} AMD		90.96 (7.28)	86.68 (5.75)
0.5 μgmL^{-1} AMD + 50 μgmL^{-1} poly IC		41.41 (3.27)	55.58 (3.04)
0.5 μgmL^{-1} AMD + 50 μgmL^{-1} poly IC	1.5	80.38 (2.30)	95.87 (5.68)

[1] RTS11 in L-15 were exposed for 24 h to high concentrations of CHX or AMD, or a combination of low, non-toxic concentration of CHX or AMD and non-toxic concentrations of poly IC.
[2] These results are representative of three similar experiments.

2.3.3. Pre or post-treatment with CHX or AMD sensitized RTS11 to Poly IC induced apoptosis

The cytotoxic actions of poly IC with either AMD or CHX did not require that the two classes of agents be present together continuously and could be elicited when they were presented separately but sequentially. Thus, RTS11 were pre-exposed to CHX or AMD for 2 h, after which the inhibitor was removed and the cells were exposed to poly IC for 20 h. Cells under these conditions died, while those exposed to each compound separately did not (Figure 2-6). The PKR inhibitor, 2-AP, totally inhibited this cytotoxicity. In dying cultures, nuclear fragmentation was apparent by H33258 staining (Figure 2-6). The numbers of fragmented nuclei in pretreated cultures were statistically different than in control cultures ($p<0.001$) and approximately 30 fold higher.

However, the numbers of apoptotic nuclei in cultures pretreated for 2h were not significantly different from cultures treated with both 50 μg mL^{-1} poly IC and 0.1 μg mL^{-1} CHX together for 24 h ($p>0.05$). These data showed that a 2 h pretreatment with 0.1 μg mL^{-1} CHX was sufficient to sensitize RTS11 to poly IC induced apoptosis. Alternatively, when poly IC (50 μg mL^{-1}) was added to cultures for 2 h and subsequently removed, over 50% of the cells were killed in cultures receiving 0.1μg mL^{-1} CHX or 0.5μg mL^{-1} AMD for 20 h. Cell viability was unchanged in cultures receiving no subsequent treatment or CHX or AMD treatment alone (data not shown).

Treating RTS11 cultures for 2 h with poly IC at 50 μg mL^{-1} failed to inhibit ^{3}H-leucine incorporation (data not shown), which is in contrast to the severe inhibition observed with CHX, as mentioned previously. Thus poly IC appears to initiate different events than the macromolecular inhibitors and these events lead to cytotoxicity only when the molecular inhibitors are subsequently added. Thus the synergism between the two classes of agents likely involves the convergence of at least two different pathways.

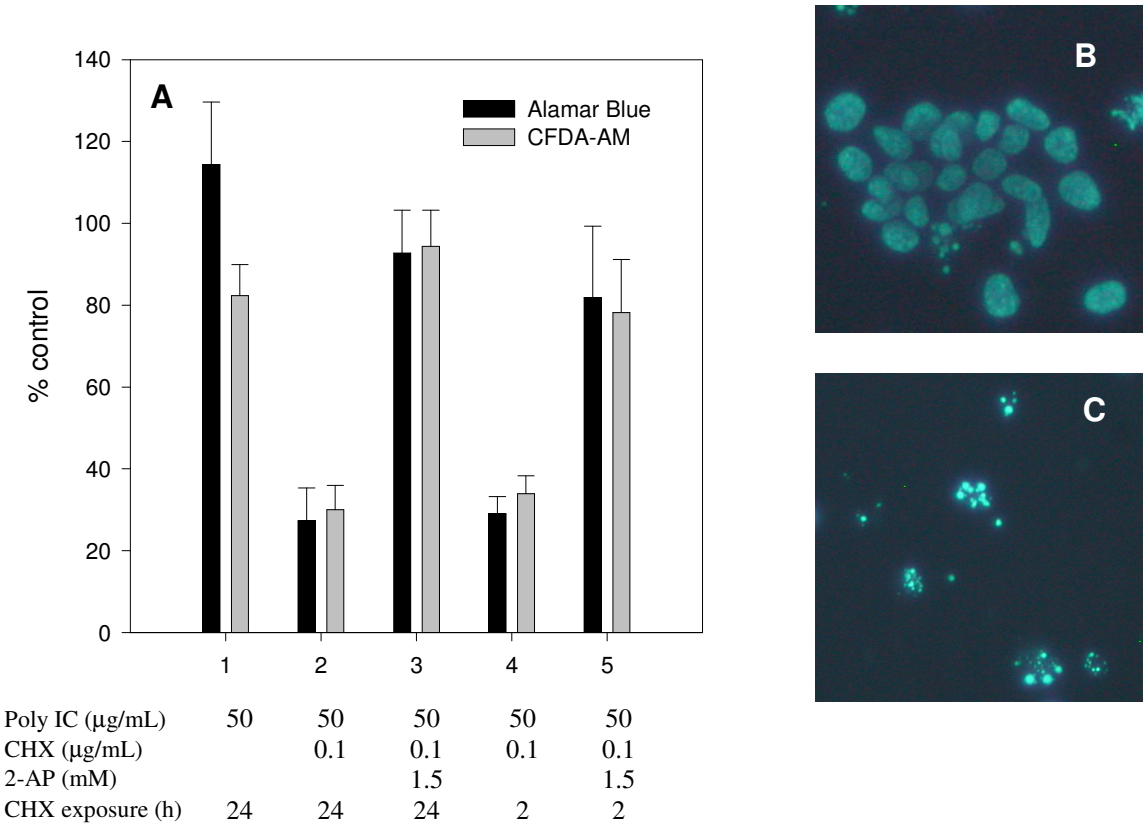


Figure 2-6. Effect of a short cycloheximide pre-treatment on the cytotoxicity of poly IC to RTS11.

The Y-axis represents cell viability, which was evaluated with alamar Blue and CFDA-AM, and expressed as a % of the control, being the inhibitor alone, or inhibitor and 2-aminopurine, while the numbers on the X-axis refer to specific combinations of experimental treatments, which are defined below each number (**A**). Bars 1 to 3 identify control cultures exposed for 24 h to poly IC either alone (1) or with CHX (2) or CHX and 2-AP (3). Bars 4 and 5 identify cultures pretreated for 2 h with CHX after which the medium with the inhibitor was removed and replaced for 20 h with medium with poly IC alone (4) or poly IC and 2-AP (5). Cells pretreated with CHX for 2h followed by 20 h in fresh media did not die (data not shown). The photographs show nuclear morphologies as revealed by Hoechst 33258 staining and fluorescence microscopy in (**B**) 50 µg ml^{-1} poly IC treated cultures and in (**C**) cultures pretreated with 0.1 µg ml^{-1} CHX for 2 h after which the inhibitor was removed and fresh medium with 50 µg mL^{-1} poly IC was added to the cells for a further 20 hours. The pictures were taken at 400X magnification.

2.3.4. Long-term exposures to poly IC alone induced cytotoxicity in RTS11

Two week exposures to poly IC alone killed RTS11 in L-15 but not in L-15 with FBS (Figure 2-7A and B). The EC_{50} values for killing by poly IC alone after a two week exposure period were 67.01 μg ml^{-1} ± 9.88 (n=3) and 15.35 μg mL^{-1} ± 5.85 (n=3) as evaluated respectively by alamar Blue and CFDA-AM. In contrast to all other treatments, which had been for 48 h or less, the EC_{50}s from the 14 day exposure period were statistically different ($p<0.002$) between alamar Blue and CFDA-AM assays. Poly IC exposures of two weeks or more did not induce cell death in CHSE-214 (Figure 2-7C), or RTG-2 (Figure 2-7 D). Similar long term exposures to single stranded RNA did not affect cell viability, indicating a dsRNA specific response (data not shown). Electrophoresis of genomic DNA from cultures exposed to poly IC for 2 weeks showed the distinctive apoptotic laddering pattern of 180 bp oligomers (Figure 2-8C). H33258 staining of cultures treated with poly IC for 14 days in L-15 demonstrate fragmented nuclei, also suggesting apoptosis (Figure 2-8A and B). However, the overall appearance of the H33258 stained culture was subtly different from H33258 stained cultures treated with AMD or CHX or poly IC plus the macromolecular inhibitors (Figure 2-2 and Figure 2-4). Firstly more nuclei appeared intact and secondly the nuclear fragments often seemed larger.

As the long-term exposures were not conducive to studies with inhibitors because they alone appeared to cause cytotoxicity over this time frame, another indication of apoptosis was sought. Thus using TEM, poly IC-treated RTS11 cultures showed cells in which nuclei were often fragmented and had condensed chromatin along the nuclear periphery (Figure 2-8E). This contrasted with control-RTS11 cultures, in which the nuclei were intact and had more uniformly distributed chromatin (Figure 2-8D).

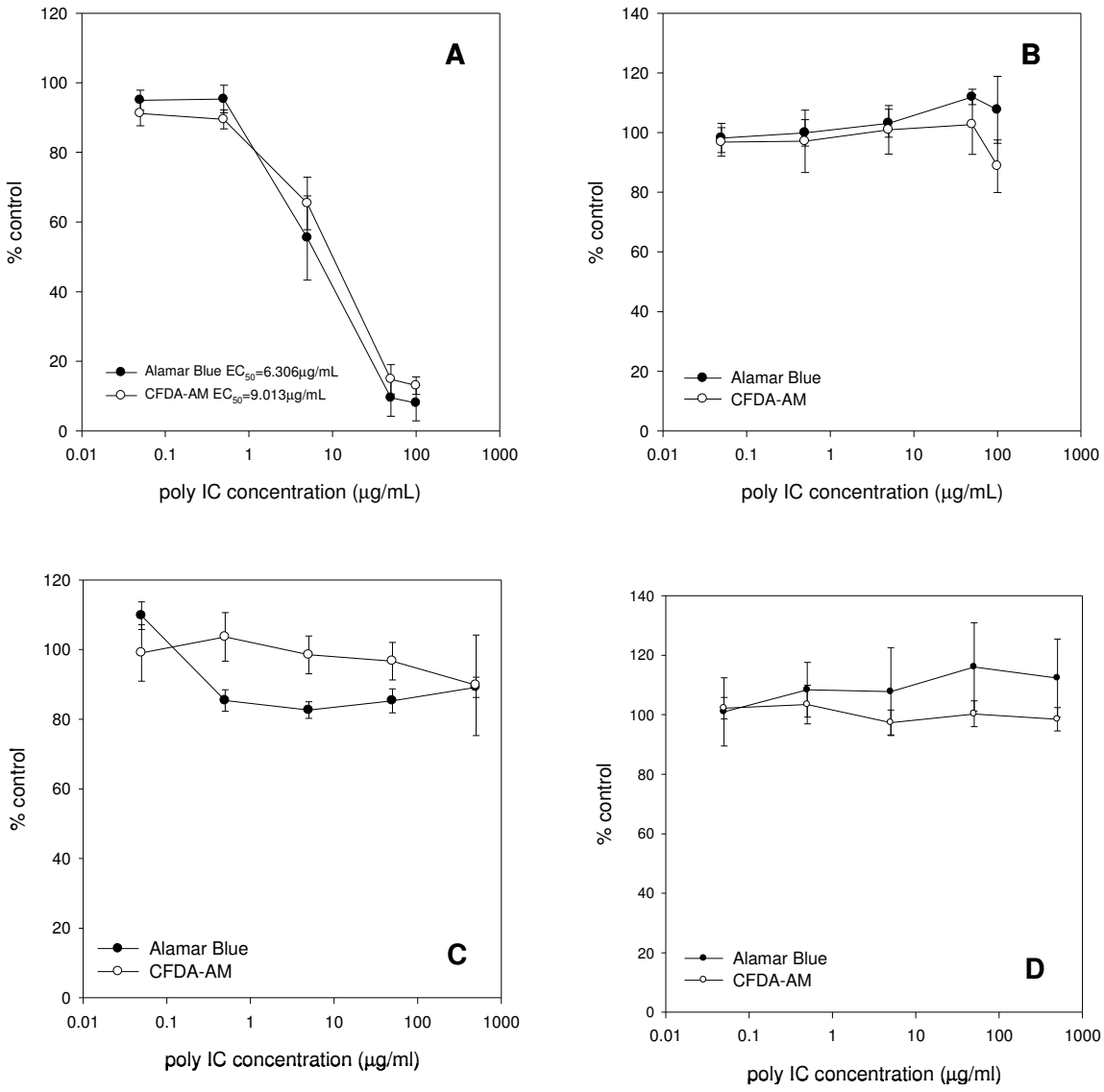


Figure 2-7. A survey of the cytotoxicity of long-term exposure to poly IC on three teleost cell lines.

Cultures of each cell line were exposed for two weeks to increasing concentrations of poly IC in L-15 with or without the addition of fetal bovine serum (FBS). Cytotoxicity was measured using two fluorescent indicator dyes, alamar Blue, which monitors metabolism, and CFDA-AM, which indicates membrane integrity. Y-axis represents cell viability, with the average relative fluorescent units (RFUs) plotted as a % of control. The control consisted of untreated cultures. The x-axis represents the concentration of poly IC in µg mL^{-1} on a logarithmic scale. (**A**) RTS11in L-15 (**B**) RTS11 in L-15 with FBS, (**C**) CHSE-214 in L-15, (**D**) RTG2 in L-15. RTS11 was found to be the only cell line to die in response to poly IC and only in L-15 without serum.

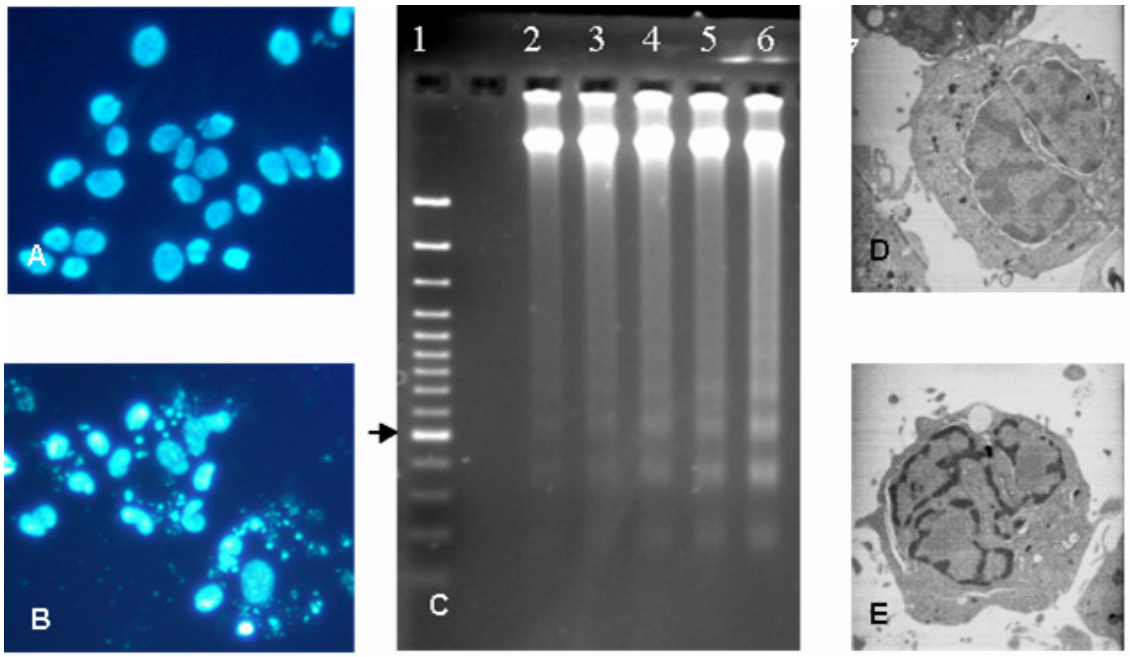


Figure 2-8. Evidence that long-term exposure to poly IC in L-15 kills RTS11 by apoptosis.

Cultures were maintained for 2 weeks in L-15 without (control) or with poly IC and then analyzed for apoptosis using three techniques. In panels **A** and **B**, cultures were stained with the nuclear fluorochrome, Hoechst 33258, and examined using a fluorescent microscope. All pictures were taken at 400X magnification. Nuclear fragmentation is evident in cultures exposed to 50 µg mL^{-1} poly IC (**B**) but not in the control (**A**). In panel **C** gDNA was extracted, run on 2 % agarose gel, and stained with EtBr. *Lane 1* is the100 bp DNA marker with the arrow identifying 500 bp. The remaining lanes contain gDNA from untreated RTS11 (lane 2), and cultures treated with 50 ng mL^{-1} poly IC (lane 3), 500 ng mL^{-1} poly IC (lane 4), 5 µg mL^{-1} poly IC (lane 5), and 50 µg mL^{-1} poly IC (lane 6). Internucleosomal fragmentation is observed in cultures treated with the higher poly IC concentrations, but not in the control cultures. In panels **D** and **E**, cultures were examined by transmission electron microscopy. Both images are at 8900X magnification. In the culture exposed to 50 µg mL^{-1} poly IC (**E**) condensation of chromatin along the periphery of the nuclear membrane and nuclear fragmentation is apparent. In the untreated culture (**D**) the chromatin appears to be diffuse and more evenly distributed throughout the intact nucleus.

2.4. DISCUSSION

A rainbow trout macrophage cell line, RTS11, in contrast to other salmonid cell lines, was susceptible under some conditions to killing by transcriptional- and translational- inhibitors, by double-stranded RNA (dsRNA), and by combinations of the two. Cytotoxicity was measured as a decline in rezasurin reduction (alamar Blue), a measure of metabolism, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) conversion to CF, an indicator of plasma membrane integrity (Dayeh et al., 2003). After 30 and 24 h exposures to either the transcriptional inhibitor, actinomycin D (AMD), or translational inhibitor, cycloheximide (CHX), respectively, RTS11 died. Conversely, no death was seen in cultures of a rainbow trout gonadal fibroblast cell line, RTG-2, or a Chinook salmon embryo cell line, CHSE-214. In RTS11 cultures without serum, the synthetic dsRNA, polyinosinic: polycytidylic acid (poly IC), killed cells in a dose-dependent manner. Cytotoxicity occurred after approximately 2 weeks of exposure to poly IC alone. When either AMD or CHX were present at concentrations that by themselves caused little or no cell death, poly IC killed within 24 h. In contrast to RTS11, RTG-2 and CHSE-214 showed no loss of viability upon being exposed to poly IC alone or in combination with the inhibitors. Thus poly IC can be cytotoxic to some fish cells as it is to some mammalian cells (Stewart et al., 1972). The sensitivity of RTS11 to poly IC allowed the mode and mechanisms underlying poly IC and cycloheximide or actinomycin D induced cytotoxicity to be investigated in a piscine system.

All treatments seemed to kill RTS11 by apoptosis, as two distinct features of apoptosis were consistently observed. H33258 staining revealed the nuclear morphology of RTS11 had the characteristic appearance of cells undergoing apoptosis. Secondly, genomic DNA was degraded into oligonucleosomal fragments, as seen with agarose gel electrophoresis. Additionally, for cultures in poly IC alone for over a 14 day period, transmission electron microscope revealed condensed chromatin along the periphery of fragmented nuclei, characteristic of apoptotic cells. For cultures in poly IC together with low concentrations of either AMD or CHX, the pan caspase inhibitor, zVAD-fmk, significantly blocked cell killing. By contrast, the killing induced by AMD or CHX alone was not inhibited by zVAD-fmk. Although caspase activation is usually considered to be a hallmark of apoptosis, recent research has identified an atypical caspase-independent apoptosis (Donovan and Cotter, 2004; Lorenzo and Susin, 2004).

Inhibitors of macromolecular synthesis

The results of this study is the first demonstration of CHX and AMD inducing apoptosis in fish cells, and supports some generalizations about this phenomenon in vertebrates. As with many cell types in mammals, some cell types in fish, as represented here by RTS11, appear to express

constitutively a long-lived apoptotic machinery that is kept suppressed by short-lived anti-apoptotic proteins. The transcriptional- and translational- inhibitors tip the balance in favor of apoptosis by preferentially reducing levels of the anti-apoptotic proteins (Tang et al., 1999; Lemaire et al., 1999; Lewis et al., 1995). In rodents and humans, AMD and CHX have been found to selectively induce apoptosis in cells *in vivo* and in cell lines *in vitro*, with cells of the immune system, such as lymphocytes (Lemaire et al., 1999), neutrophils (Tsuchida et al., 1995) and macrophages (Lewis et al., 1995), being particularly sensitive. The relative resistance of some cells to killing by AMD and CHX is not due to these agents failing to fully inhibit transcription and translation in these cells (Chang et al., 2002). The apparent sensitivity of immune cells in mammals might also be true in fish because RTS11 was killed by CHX and AMD under conditions where the fibroblast (RTG-2) and epithelial (CHSE-214) cells were not. RTS11 could differ from these cell lines in the type, stability and/or amount of anti-apoptotic protein(s) restraining a constitutive apoptotic apparatus.

Two features of CHX- and AMD-induced RTS11 apoptosis were unique. For the first time in any system, the induction of apoptosis by CHX or AMD has been shown to be blocked by 2-aminopurine (2-AP), which is best characterized as an inhibitor of PKR but possibly could inhibit other tyrosine kinases (Hu and Conway, 1993; Essbauer and Ahne, 2002). A possible explanation for this result is suggested by recent studies on the fungal translational inhibitor, deoxynivalenol (DON) (Zhou et al., 2003). DON binds to ribosomes, causing a ribotoxic stress response that includes activation of mitogen-activated protein kinases (MAPKs) and leads to apoptosis. PKR is thought to be a transducer of the ribotoxic stress response that is initiated by DON and other translational inhibitors (Zhou et al., 2003). Therefore, as well as causing a decline in the levels of anti-apoptotic proteins, CHX and AMD might induce RTS11 apoptosis by causing a ribotoxic stress response mediated through PKR. The second unique feature of CHX- and AMD-induced RTS11 apoptosis was that caspase 3 was not a mediator as zVAD-fmk failed to block killing. With mammalian cells, caspase 3 mediated CHX-induced apoptosis of murine B cells (Lemaire et al., 1999). However, serine proteases as well as caspases appeared to be involved in the induction of apoptosis in the human macrophage cell line U937 by the translational inhibitors, anisomycin and ricin (Kageyama et al., 2002). Possibly a serine protease mediates CHX- and AMD- induced RTS11 apoptosis.

Poly IC with inhibitors of macromolecular synthesis

At concentrations that caused very little or no cell death, CHX and AMD sensitized RTS11 to killing by poly IC through a mechanism mediated by PKR. The killing was completely blocked

by the presence of 2-AP, which is primarily an inhibitor of PKR. Poly IC could act through PKR to induce apoptosis in CHX- or AMD- sensitized RTS11 in at least two ways: modulating the death receptor pathway and/or causing 'ribotoxic stress'. In many mammalian cell systems, dsRNA-stimulated PKR-mediated apoptosis has been associated with a modified death receptor pathway (Jeffrey et al., 2002). Several reports indicate that dsRNA-induced apoptosis occurs *via* the Fas-associated death domain (FADD), independent of death ligand/ death receptor interactions (Balachandran et al., 1998; Gil and Esteban, 2000a; Gil and Esteban, 2000b). Even with almost complete protein synthesis inhibition, PKR could tip the balance in sensitized cells towards apoptosis by selectively up regulating pro-apoptotic factors in this pathway (Balachandran et al., 1998; Decker, 1992). Therefore PKR-mediated changes in protein expression in combination with decreased levels of protective proteins could be responsible for RTS11 apoptosis.

Alternatively or additionally, poly IC could activate RNase L, causing rRNA cleavage and ribotoxic stress (Li et al., 2004), which would trigger an apoptotic pathway mediated by PKR (Zhou et al., 2003). CHX or AMD treatments or pretreatments could allow this pathway to be expressed by reducing the level of anti-apoptotic factors and/or by acting additively or synergistically with poly IC to cause ribotoxic stress.

Several macrophage-specific cellular differences, possibly working in combination, could explain why AMD or CHX treatment sensitized RTS11 but not other cell lines to poly IC. One could be the nature of the anti-apoptotic proteins expressed. In some circumstances CHX and AMD have been reported to sensitize cells to apoptosis *via* the FADD locus by rapidly decreasing the level of cellular FADD-like interleukin 1-converting enzyme-inhibitory protein (cFLIP) (Fulda et al., 2000). cFLIP inhibits the formation of a DISCs (death-inducing signaling complexes) (Scaffidi et al., 1999). Macrophages express high levels of cFLIP (Perlman et al., 1999), and being macrophage-like, RTS11 may also express high levels of this short-lived protective protein. Another difference could be in the capacity of different cell lines to respond to dsRNA and the nature of the responses initiated. For example, RTS11 might take up dsRNA better than other cell lines or express more toll-like receptor 3, which in mammalian macrophages is a membrane receptor for dsRNA (Alexopoulou et al., 2001). Additionally, dsRNA might trigger one or more pro-apoptotic pathways in RTS11, perhaps by activating RNase L or interferon response factor 3 (IRF-3) (Hsu et al., 2004).

Poly IC alone

The killing of RTS11 by long-term exposure to poly IC alone is rare in the mammalian cell literature. Nearly all reports of mammalian cells treated with poly IC commonly 'co-treat' with macromolecular synthesis inhibitors or cytokines such as interferons. These co-treatments kill cells by apoptosis after exposure times of 48 h or less (Der et al., 1997; Kalai et al., 2002). In mammals, dsRNA stimulates production of interferon, which in turn can induce the synthesis and release of TNFα (Chawla-Sarkar et al., 2001). Both IFN-α/β and TNFα act together with dsRNA to cause apoptosis (Yeung et al., 1996). In RTS11, poly IC up regulates IL-8 and TNF-α expression (Laing et al., 2001; Laing et al., 2002b). More recently, interferon has also been cloned from RTS11 (GenBank accession nos. **AJ582754**, and **AJ580911**). As the medium was not changed during the 2 week exposure, poly IC could cause the slow accumulation in the medium of factors, such as interferon and TNFα, ultimately reaching levels that can act together with the poly IC to cause RTS11 apoptosis. The killing likely occurred asynchronously so that other RTS11 began phagocytizing some apoptotic cells, accounting for the slightly different appearance of these cultures after H33258 staining. The failure of poly IC to induce RTS11 death in FBS-containing medium could be due to serum survival factors over-riding any killing factors produced by RTS11. The lack of cell death in poly IC-treated RTG-2 cultures could be because these cultures do not either accumulate apoptosis-inducing cytokines or respond to them. CHSE-214 would not be expected to die because this cell line has long been observed to be unresponsive to exogenously added dsRNA (MacDonald and Kennedy, 1979; Jensen et al., 2002b).

In summary several mechanisms appear to trigger apoptosis in RTS11. As apoptosis is implicated in so many disease processes in mammals, the current results should help begin delineating mechanisms of cell death in fish and how they interact, ultimately leading in the long term to developing methods of improving fish health.

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Chapter 3

Gliotoxin-induced cytotoxicity in three salmonid cell lines: cell death by apoptosis and necrosis

ABSTRACT

Epithelial (CHSE-214), fibroblast (RTG-2) and macrophage (RTS11) cell lines from Chinook salmon and rainbow trout were tested for their sensitivity to gliotoxin, a fungal metabolite. Gliotoxin treatment for 6 or 24 h caused cell viability to decrease in a dose-dependent manner, with effective concentrations (EC_{50}s) being similar for the three cell lines but varying with exposure time. Under some exposure conditions, hallmarks of apoptosis were detected. Apoptosis was evaluated by the appearance of fragmented nuclei upon H33258 staining and of genomic DNA laddering into 180 bp oligomers. Gliotoxin induced cell detachment in RTG-2 and CHSE-214 cultures, under some conditions. These were the only cultures of these two cell lines in which apoptosis was detected, and apoptotic cells appeared more frequent in the detached population. At the highest concentration, 15 μM, the cells died by an alternative mode, likely necrosis. By contrast, in RTS11 cultures cell detachment was not observed, and apoptosis occurred over a wider concentration range, even 15 μM, reaching levels of over 90 %. The preferential death by necrosis for epithelial cells (CHSE-214) and by apoptosis for macrophages (RTS11) could be a beneficial host response to gliotoxin-producing fungi, leading respectively to the development and then resolution of inflammation.

3.1. INTRODUCTION

Although human and rodent cell lines have been invaluable tools for studying apoptosis or programmed cell death in mammals, the capacity of fish cell lines for apoptosis has been largely unexplored, despite their growing use in toxicology and ecotoxicology (Bols et al., 2004; Segner, 1998; Bols et al., 2005). Apoptosis is a fundamental process of normal animal development and physiology and is implicated in many disease processes and toxicological responses (Jones and Gores, 2005). Several physical treatments and a rapidly expanding list of chemical and biological agents induce apoptosis in mammalian cell lines. Yet some mammalian cell lines do not undergo apoptosis but die by necrosis (Kolenko et al., 1999). For fish cell lines, apoptosis has been best characterized in response to a few viruses (Chiou et al., 2000; Hong and Wu, 2002), although recently a bullhead fibroblast cell line has been shown to express several apoptosis-related genes (Busch et al., 2004). Knowing whether a particular fish cell line is able to undergo apoptosis would help in choosing the cell line(s) appropriate for studying the toxic mechanisms of ecotoxicants and for monitoring them in environment samples. Therefore, several salmonid cell lines have been studied for their response to gliotoxin, which is known to kill mammalian cell lines by apoptosis and is sometimes used as a positive control (Kashimoto et al., 2003).

Gliotoxin is an epipolythiodioxopiperazine fungal metabolite that has various bioactivities, the most studied being immunosuppression and induction of apoptosis (Waring and Beaver, 1996). Immunosuppression appears to be mediated by the ability of gliotoxin to inhibit nuclear factor (NF) -κB. Apoptosis induced by gliotoxin is caspase dependent but the mechanism of cell death is still incompletely understood (Zhou et al., 2000). Cells in primary cultures and *in vivo* have been shown to die by apoptosis in response to gliotoxin (Waring and Beaver, 1996), but a growing body of work has been amassed using cell lines. Gliotoxin has been shown to induce apoptosis in fibroblast, epithelial and macrophage-like cell lines from mice, rats, pigs and humans (Kashimoto et al., 2003; Kweon et al., 2003; Upperman et al., 2003; Waring and Beaver, 1996; Zhou et al., 2000). Recently, the cytotoxicity of gliotoxin to an insect cell line was demonstrated (Fornelli et al., 2004).

As well as being a reagent in cell biology research, gliotoxin is of interest as a toxic or virulence factor of fungi that cause diseases in humans and other animals (Waring and Beaver, 1996). For example, gliotoxin from *Aspergillus fumigatus* has been implicated in gut barrier dysfunction in humans and in respiratory disease in turkeys respectively (Richard, 1997; Upperman et al., 2003). A role for gliotoxin in fish diseases has yet to be reported but *A.*

fumigatus has been isolated from the intestinal tract of a marine fish (Numata et al., 1992) and fungal infections cause important economic losses in salmon aquaculture (Meyer, 1991).

In this study, the cytotoxicity of gliotoxin to epithelial (CHSE-214), fibroblast (RTG-2) and macrophage (RTS11) cell lines from Chinook salmon and rainbow trout (RT) was undertaken for three purposes. The first was to determine the capacity of these cell lines to undergo apoptosis in order that this information could be used to choose the appropriate cell lines for studies in toxicology and ecotoxicology. For this aim, the study has been performed in cultures with and without fetal bovine serum (FBS). Although commonly used to grow fish cell lines, FBS can modify responses to toxicants (Bols et al., 2005), but these three cell lines can survive under some conditions for a week or more without FBS (Lee et al., 1988; Barlian et al., 1993; Pagniello et al., 2002), making comparisons of responses with and without FBS possible. The second purpose was to study for the first time the cytotoxicity of gliotoxin to fish cells, which to date has been studied only with mammalian cells, but which potentially could be a product of fish fungal pathogens. The final purpose was to compare the mode of cell death induced by gliotoxin in the three different cell line types: epithelial, fibroblast and macrophage. Although killing the three cell lines, gliotoxin preferentially killed the epithelial and fibroblast cell lines by necrosis and the macrophage cell line by apoptosis. The work represented in this chapter has been published (DeWitte-Orr and Bols, 2005).

3.2. MATERIAL AND METHODS

3.2.1. Cell Culture

The cell lines were Chinook salmon embryo (CHSE-214), rainbow trout gonadal (RTG-2), and rainbow trout monocyte/macrophage (RTS11). RTG-2 and CHSE-214 were obtained from the American Type Culture Collection (ATCC), whereas RTS11 was developed in this laboratory (Ganassin and Bols, 1998). The routine growth of these cells lines has been described in detail previously (Bols and Lee, 1994; DeWitte-Orr et al., 2005). Briefly, cultures were maintained at 18-21 °C in Leibovitz's L-15 supplemented with fetal bovine serum (FBS) and penicillin G/streptomycin sulfate. These items were purchased from Sigma (St. Louis, MO). Cultures were grown in 25 and 75 cm^2 plastic tissue culture flasks from Nunc (Roskilde, Denmark). For experiments, slide flasks (Nunc) as well as 6 and 96 well plates (Falcon/Becton-Dickenson, Franklin Lakes, NJ) were used.

3.2.2. Cytotoxicity Assay

Plating and dosing

Cells were seeded into 96 well plates, in L-15 growing medium with or without the FBS supplement. CHSE-214 and RTG-2 cells were seeded at 4.0 x 10^4 cells per well while RTS11 were seeded at 1.5 x 10^5 cells per well. Cells were allowed to settle and reattach for 24 h at their normal growing temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of gliotoxin (Sigma). For the potentially toxic effects of the carrier ethanol, cells were treated with an equal volume of the carrier itself as a control. After a specific incubation time, cultures were evaluated for cytotoxicity. In no cases was the solvent used at a concentration that was cytotoxic. Results were calculated as a percent of the carrier-treated control cells.

Measuring cytotoxicity

Two fluorescent indicator dyes, alamar Blue™ (Medicorp, Montreal, PQ) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Molecular Probes, Eugene, OR) were used to evaluate cell viability. Viable cells cause the reduction of alamar Blue dye resulting in a chemical change from a non-fluorescent, blue form (resazurin) to a fluorescent red form (resorufin)(O'Brien et al., 2000). CFDA-AM, which is also taken up by live cells, is hydrolyzed by non-specific intracellular esterases to yield the fluorescent form, carboxyfluorescein. These esterases are only active within the confines of a cell; therefore, CFDA-AM can be used to

monitor cell membrane integrity (Schirmer et al., 1997). The levels of fluorescence are measured using a microplate spectrofluorometer. Alamar Blue and CFDA-AM assays were performed as previously described (Ganassin et al., 2000). Briefly, cells were seeded in a 96 well plate as described above. After 24 h incubation at normal growing temperature, the cells were dosed with gliotoxin. After further incubation for the described amount of time, at normal growing temperatures, the media was removed and the two fluorescent dyes were added to the wells, diluted in a minimal salt solution called L-15ex. The cells were incubated with the dyes for 60 minutes before the microwells were read with a fluorometric plate reader (Molecular Devices SpectraMax). Excitation and emission wavelengths were 530 and 595 nm for alamar Blue and 485 and 530 nm for CFDA-AM respectively.

3.2.3. Determining Apoptosis

For evidence of apoptosis, cultures were evaluated for nuclear fragmentation as assessed by Hoechst staining and genomic DNA fragmentation. Unless otherwise indicated, RTS11 cells were seeded at $4.0x10^6$ cells in a $9cm^2$ slide flask for Hoechst staining and $7.0x10^6$ cells in a $12.5cm^2$ tissue culture treated flask for genomic DNA (gDNA) extraction. RTG-2 and CHSE-214 were seeded at $1.0x10^6$ cells in a $9cm^2$ slide flask and $1.6x10^6$ cells in a $12.5cm^2$ tissue culture treated flask. Cells were seeded in L-15 growing medium, and incubated for 24 h at normal growing temperatures. The cells were then dosed with gliotoxin and incubated at normal growing temperatures for the required amount of time.

Hoechst 33258 stain

After the indicated exposure time, cultures were fixed by adding an equal volume of Carnoy's fixative (methanol: glacial acetic acid, 3:1), which was prepared fresh with each use, to existing media, exposing the cells for 2 minutes. Following the initial fixation, the media and fixative was removed and the cells were exposed to fresh fixative twice, each incubation being 5 minutes. In cultures where gliotoxin induced cell detachment, the detached cells were collected in the first fixative/media incubation and centrifuged in a swinging bucket rotor centrifuge at 444xg for 5 minutes to collect the cells. These collected cells were fixed twice with Carnoy's fixative, with a 5 minute centrifugation between fixing. Cells were then adhered to a glass slide using a cytocentrifuge (Shandon Elliott) at 500 rpm for 5 minutes.

The adherent and cytospun detached cell slides were air-dried and stained with 0.5 μg/mL Hoechst (H) 33258 (Riedel-de Haen Ag Seetz-Hannover) for 10 minutes, followed by a rinse in deionized water. After drying, a coverslip was mounted onto the slide with PBS:glycerol (1:1). The fluorescent nuclei were visualized using a fluorescence microscope with a ultra-violet (UV)

filter. A total of 300 nuclei in three randomly chosen fields on each slide were scored as being intact or having the characteristic fragmented appearance of apoptotic cells. In many cases the scoring of the detached cells was difficult because the RTG-2 and CHSE-214 cells usually detached in clumps or sheets, which sometimes folded back on themselves during collection onto slides, making the visualization and scoring of individual nuclei difficult. Thus the percentage of apoptotic nuclei among the detached cells was not calculated.

DNA fragmentation ladder

After a specified treatment, all cells, whether adherent or detached, were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit (Sigma), which is based on column affinity technology. The genomic DNA was eluted from the column using 100 µL Milli-Q water, and 35 µL of this DNA was resolved by electrophoresis on a 2% (w/v) agarose gel for 4 h at 60V. The DNA ladders were visualized by staining gels with 0.5 µg/mL ethidium bromide and photographed under a UV transillumination.

Transmission Electron Microscopy

RTS11 were seeded in 25cm^2 tissue culture treated flasks at 10.5x10^6 cells per flask in L-15 growing medium. After 24 h incubation at 18^{o}C to allow reattachment, the cells were dosed with 1.5 µM gliotoxin, and incubated again at 18^{o}C for 2h. At this time the gliotoxin treated cells began to show signs of blebbing and were collected and pelleted using a swinging bucket rotor centrifuge. The cell pellet was resuspended in 0.2 M phosphate buffer (pH 7.2) for 30 minutes. The phosphate buffer was then replaced and cells were incubated overnight in a 0.2M phosphate buffer with 2.5% gluteraldehyde (pH 7.2). The cells were then washed three times with 0.2 M phosphate buffer (pH 7.2) and incubated in 1% OsO_4 solution for 2 h. The OsO_4 solution was then removed by washing the cells twice in distilled water for 30 minutes. The cells were then dehydrated in a series of graded acetone (to 100%). The dehydrated cells were embedded in Epon-Araldite, in Beem capsules. Sections of the embedded cells were taken, at 60-90nm, using an ultramicrotome (Reichert Ultracut E). These sections were placed on 3mm copper grids and stained with freshly prepared lead citrate for 10 minutes, after which the cells were rinsed with distilled water. The cells were then stained with freshly prepared saturated uranyl acetate stain in 70% ethanol. To remove the stain, the sections were washed in 70% ethanol and dried. Prepared sections were viewed using a transmission electron microscope (Philips CM10).

Data analysis

For alamar Blue and CFDA-AM assays, cytotoxicity was indicated by a decline in fluorescence units (FUs) for experimental cultures relative to control cultures. FUs for culture wells with no cells were constant and subtracted from FUs for experimental and control cultures. For graphical presentation, the results were plotted using SigmaPlot (Jandel Scientific), and EC_{50} values were determined using the logistic function option in SigmaPlot for dose-response curves (Dayeh et al., 2003). EC_{50} values for the two different assays and for different exposure conditions were compared by an unpaired t-test. EC_{50} values between the three cell lines were compared by a one-way analysis of variance (ANOVA). For the number of apoptotic nuclei in cultures after different gliotoxin exposures, an ANOVA with Dunnett's post test was performed ($p \leq 0.05$). All statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com).

3.3. RESULTS

Cultures of macrophage (RTS11), fibroblast (RTG-2) and epithelial (CHSE-214) cell lines were exposed to gliotoxin at different concentrations, in the basal medium, Leibovitz L-15, with or without fetal bovine serum (FBS), for usually 6 or 24 h, and examined for changes in morphology, viability, and mode of cell death.

3.3.1. Morphology

As judged by light microscopy, gliotoxin caused morphological changes in cultures, which varied with the exposure time, the presence of FBS, and cell line. Membrane blebbing was seen as early as 1 h in RTS11 cultures but not for several hours later with the other two cell lines (data not shown). In RTS11 cultures with or without FBS, cells remained attached to the growth surface for exposures of up to 24 h at all gliotoxin concentrations tested. By contrast, in RTG-2 and CHSE-214 cultures, cells largely remained attached under all conditions after 6 h but some were detached by 24 h under restricted conditions. Detachment was observed at 0.15 and 1.5 μM in L-15 without FBS and at 1.5 and 15 μM with FBS. Without FBS the majority of cells remained attached, whereas with FBS, nearly all cells detached, leaving just a few adherent cells. The detached cells were often seen in clumps or sheets. After 24 h at 15 μM in L-15 without FBS, RTG-2 and CHSE-214 cells remained attached and had a distinctively different morphology than the control cells (Figure 3-1) that might be best described as shrunken with lengthy cellular extensions. The results from the Hoechst staining highlight differences in nuclear morphology between cell lines; RTS11 nuclei were fragmented indicating apoptosis, while RTG-2 and CHSE-214 were intact suggesting an alternative form of cell death, perhaps necrosis.

3.3.2. Viability after 6 h gliotoxin exposures

Whether in L-15 with or without FBS, the three cell lines showed a dose-dependent decline in viability after a 6 h exposure to gliotoxin (Figure 3-2). Viability was measured as a decline in rezasurin reduction (alamar Blue, AB), which indicates an impairment of metabolism, and in the conversion of 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) to CF, which measures esterase activity and indicates an impairment of plasma membrane integrity. The EC_{50}s from the dose-response curves were used to compare viability measurements. Metabolism as measured by AB was more sensitive to impairment by gliotoxin at 6 h than plasma membrane integrity as indicated by CFDA-AM in the three cell lines without FBS and in RTS11 and

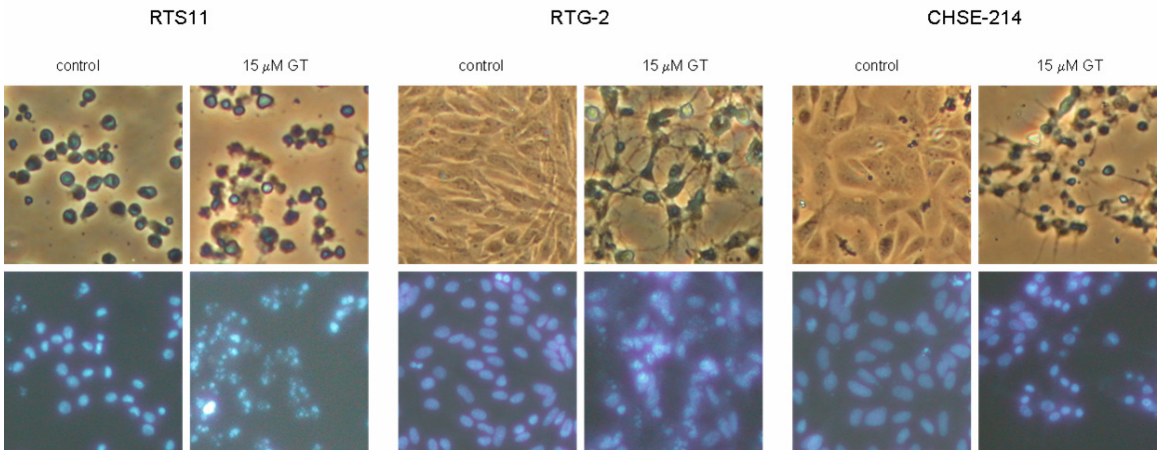

Figure 3-1. Effect of gliotoxin on the morphology of three salmonid cells lines.

RTS11, RTG-2 and CHSE-214 cultures were treated for 24h in serum free L-15 with 15 μM gliotoxin or carrier (EtOH) alone before being fixed, stained with the fluorochrome H33258, which stains nuclear DNA, and examined by phase contrast and fluorescence microscopy. Phase contrast photographs of the cultures are shown in the top panels and the corresponding fluorescence pictures are shown in the bottom panels. All pictures were taken at 400X magnification.

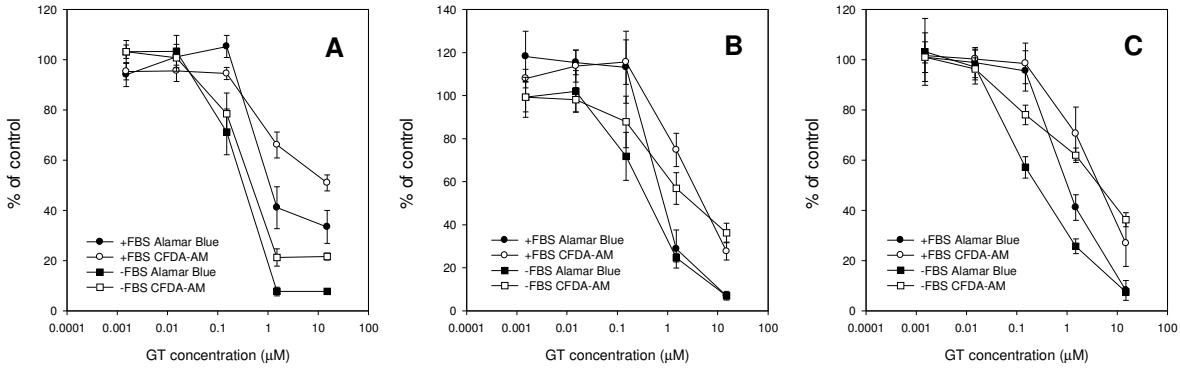

Figure 3-2. Effect of 6 h exposures to gliotoxin on the viability of three salmonid cells lines.

RTS11 (**A**), RTG-2 (**B**), CHSE-214 (**C**) were exposed to gliotoxin in L-15 with or without FBS. Cell viability was measured using two fluorescent indicator dyes, alamar Blue (AB) and CFDA-AM, and was expressed on the Y-axis as a % of control cultures treated with the carrier (EtOH) alone. The x-axis represents the concentration of gliotoxin in μM on a logarithmic scale. For each concentration, six wells were exposed and the mean and standard deviations are graphed. This graph is representative of three similar experiments (see Table 3-1).

CHSE-214 with FBS (t-test, $p < 0.05$; Table 3-1). Regardless of the measure of cell viability, the EC_{50}s were similar between the cell lines (ANOVA, $p > 0.05$). However, for RTS11, EC_{50}s were higher in L-15 with FBS than in L-15 without FBS (t-test, $p < 0.05$), but for the other two lines, EC_{50}s were similar in L-15 with or without FBS (t-test, $p > 0.05$).

3.3.3. Mode of cell death after 6 h gliotoxin exposures

During 6 h treatments with gliotoxin, RTS11 cultures demonstrated several significant hallmarks of apoptosis, whereas these were absent in RTG-2 and CHSE-214 cultures. When genomic DNA (gDNA) from control and gliotoxin-treated cultures were run on a 2% agarose gel in order to ascertain whether the DNA had been cleaved into 180bp oligomers, gDNA laddering was evident in gliotoxin-treated cultures of RTS11 but not in RTG-2 and CHSE-214 (Figure 3-3). Hoechst (H) 33258 staining revealed that many cells in RTS11 cultures had fragmented nuclei, which is another common characteristic of apoptosis. Even at high gliotoxin concentrations, the vast majority of RTG-2 and CHSE-214 nuclei remained intact (Figure 3-4). In correspondence with the cell viability data, the total number of apoptotic nuclei was lower in gliotoxin treated RTS11 cultures with FBS. For example, approximately 60% of the nuclei in RTS11 cultures treated with 1.5 μM gliotoxin for 6h in L-15 without serum were apoptotic while those treated in L-15 with serum had only about 20% (Figure 3-5). The fragmentation of RTS11 nuclei was confirmed by transmission electron microscopy (TEM). RTS11 cultures treated with gliotoxin for 2h clearly showed cellular blebbing, nuclear fragmentation and compartmentalization of nuclear material (Figure 3-6B). Cells in carrier treated control cultures had single intact nuclei, with evenly distributed chromatin, as well as an intact, continuous cellular membrane (Figure 3-6A).

3.3.4. Viability after 24 h gliotoxin exposures

Measurement of cell viability after 24 h gliotoxin treatments revealed several subtle changes from 6 h (Table 3-1, Figure 3-7). For each cell line, impairment of membrane integrity as measured with CFDA-AM and of metabolism as measured by AB was now the same (t-test, $p > 0.05$; Table 3-1). The exception was CHSE-214 in no serum, where the AB EC_{50} was significantly lower than the CFDA-AM EC_{50} (Table 3-1). The dose-response curves for each cell line shifted to the left, with 24 h EC_{50}s being lower than 6 h EC_{50}s (t-test, $p < 0.05$) in all cases, except for AB on CHSE-214 in FBS (t-test, $p > 0.05$). The 24 h EC_{50}s for the three cell lines continued to be similar to each other except for viability assessments with CFDA-AM on cultures

Table 3-1. Effect of gliotoxin (GT) on the viability of three salmonid cell lines*.

	Exposure conditions		Mean EC_{50}s from two viability assays	
Cell line	time (h)	% FBS	Alamar Blue μM ± SD (n =3)	CFDA-AM μM ± SD (n =3)
RTS11 (macrophage)	6	15	1.35 ± 0.06	1.679 ± 0.195
	6	0	0.24 ± 0.04	0.471 ± 0.133
	24	15	0.45 ± 0.07	0.475 ± 0.072
	24	0	0.11 ± 0.01	0.111 ± 0.002
RTG-2 (fibroblast)	6	10	2.00 ± 0.74	10.55 ± 9.27
	6	0	1.48 ± 1.10	5.11 ± 3.31
	24	10	0.31 ± 0.13	0.34 ± 0.14
	24	0	0.12 ± 0.01	0.12 ± 0.02
CHSE-214 (epithelial)	6	10	1.65 ± 0.65	4.34 ± 0.73
	6	0	0.94 ± 0.32	8.01 ± 5.16
	24	10	0.73 ± 0.62	1.69 ± 1.37
	24	0	0.21 ± 0.18	0.99 ± 0.11

*see text for statistical comparison

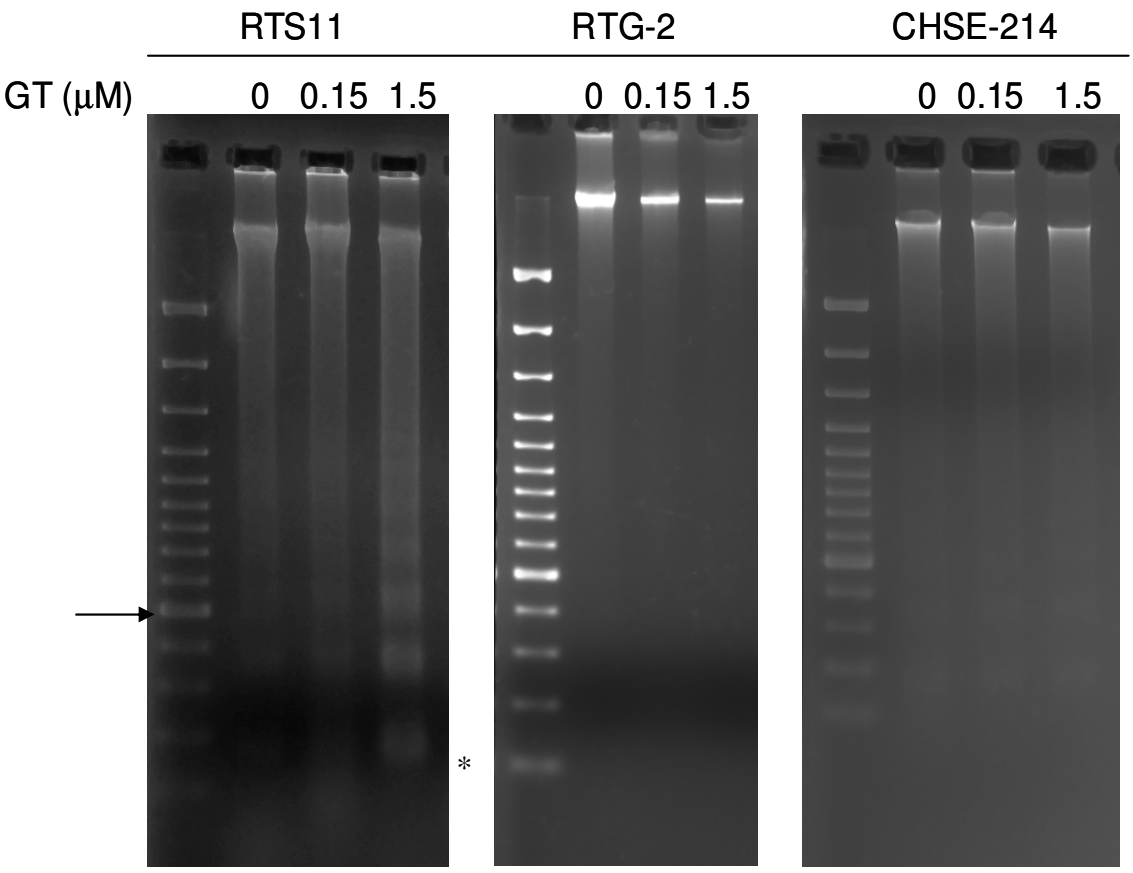

Figure 3-3. Effect of 6 h gliotoxin exposures on the integrity of genomic DNA in three salmonid cell lines.

Genomic DNA was extracted after RTS11, RTG-2, and CHSE-214 cultures had been exposed to varying gliotoxin concentrations in L-15 with FBS and run on a 2% agarose gel. The DNA marker is in 100bp increments with an arrow at 500bp. An asterisk identifies the position of the 180 bp fragment. Only RTS11 demonstrated the typical 180 bp oligomers distinctive of apoptosis.

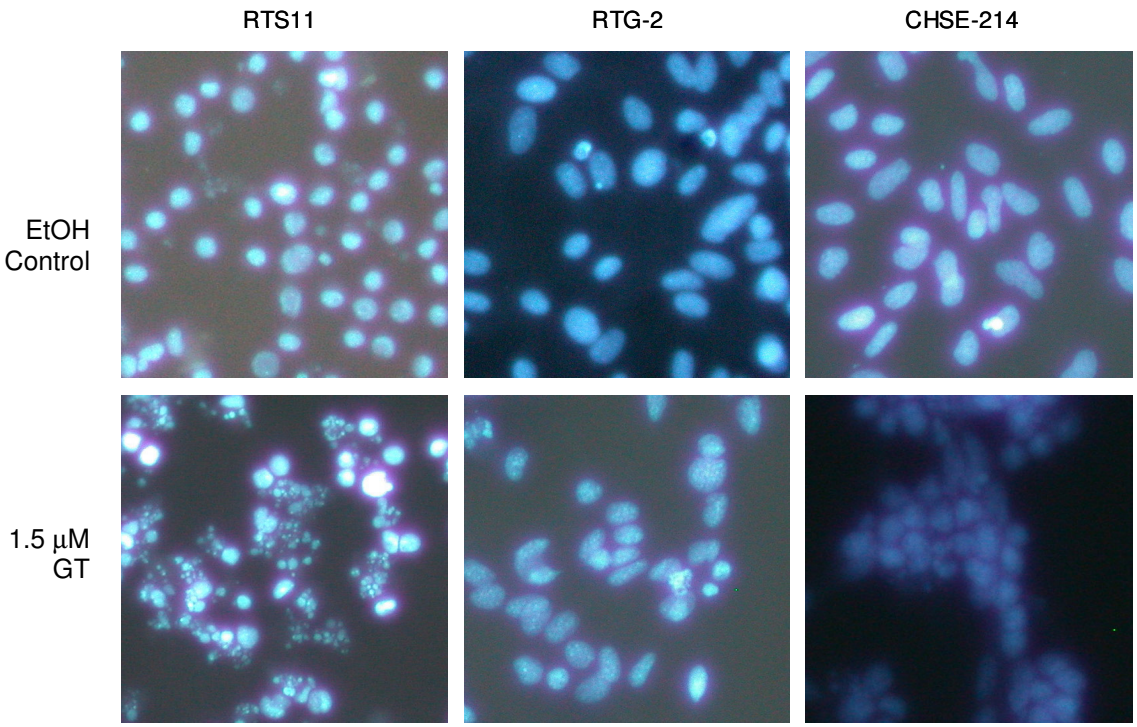

Figure 3-4. Effect of 6 h gliotoxin exposures on the nuclear morphology of three salmonid cell lines as revealed by H33258 staining and fluorescence microscopy.

RTS11, RTG-2, and CHSE-214 cultures in serum free L-15 were exposed to 1.5 µM gliotoxin or EtOH alone (control). After 6 h, they were fixed, stained with the fluorochrome H33258, which stains nuclear DNA, and examined by fluorescence microscopy. The top panel shows intact nuclei in control cultures. The bottom panel shows fragmented nuclei only in the gliotoxin-treated RTS11 culture. All pictures were taken at 400X magnification.

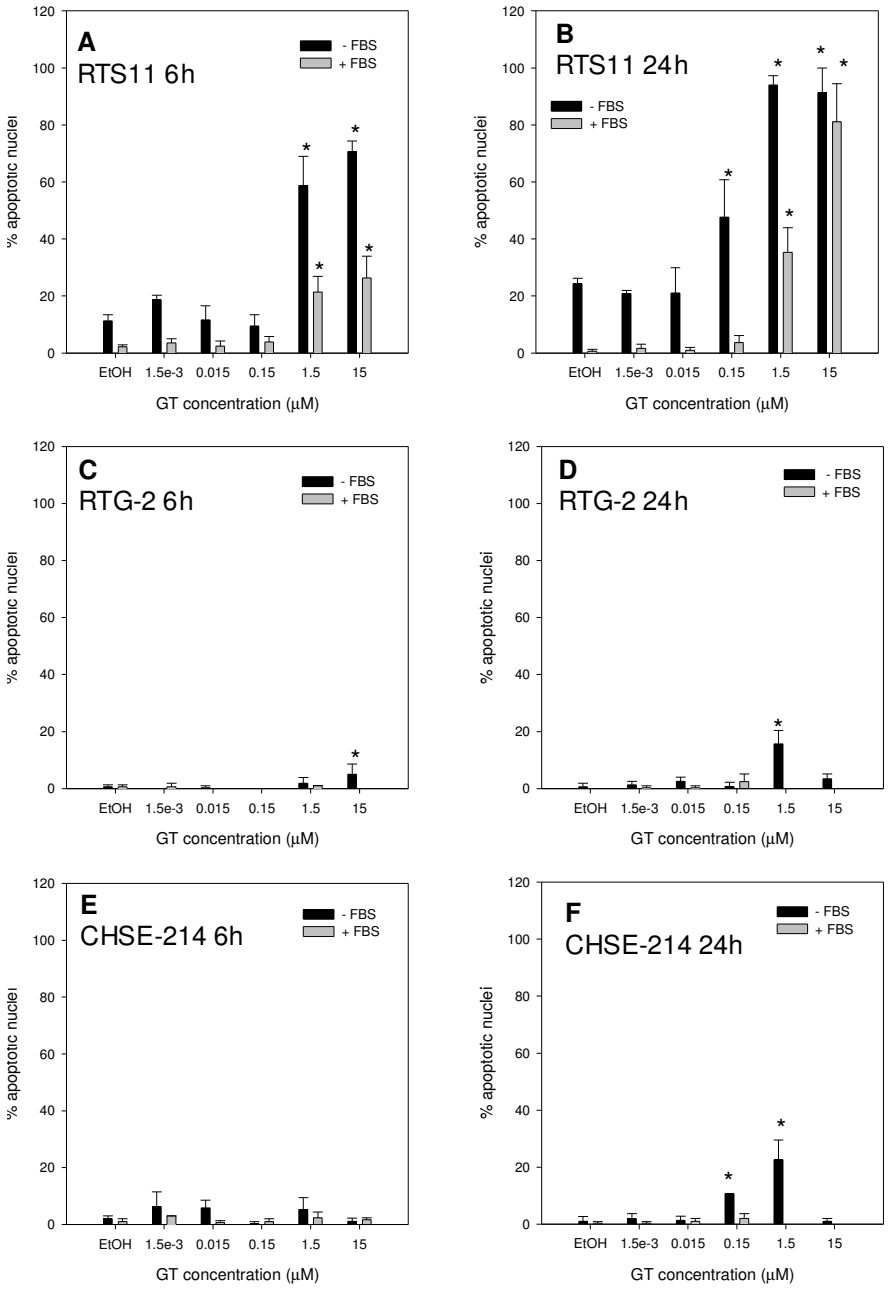

Figure 3-5. Percentage of fragmented nuclei in the cells remaining adherent in cultures of three salmonid cell lines treated with increasing concentrations of gliotoxin (GT).

The cell lines were RTS11 (**A** & **B**), RTG-2 (**C** & **D**), and CHSE-214 (**E** & **F**) and were exposed for either 6 h (**A**, **C** & **E**) or 24 h (**B**, **D** & **F**) in the presence [stippled bar] or absence [solid bar] of fetal bovine serum (FBS). After H33258 staining, cultures were viewed by fluorescence microscopy and 300 randomly chosen nuclei were scored for fragmentation. The results were analyzed by a one-way ANOVA, and if significant, the values were compared to the values in control cultures by Dunnett's test. An asterisk identifies the cultures statistically significant from control cultures ($p<0.05$). The % of fragmented nuclei is plotted. A considerable number of cells were detached in RTG-2 (**C**) and CHSE-214 (**E**) cultures exposed to 0.15 and 1.5 µM GT for 24 h in the absence of FBS, and these detached cells were not considered in the calculation of the % fragmented nuclei. All cells detached in RTG-2 and CHSE-214 cultures after 24 h exposure to 1.5 and 15 µM GT in presence of FBS.

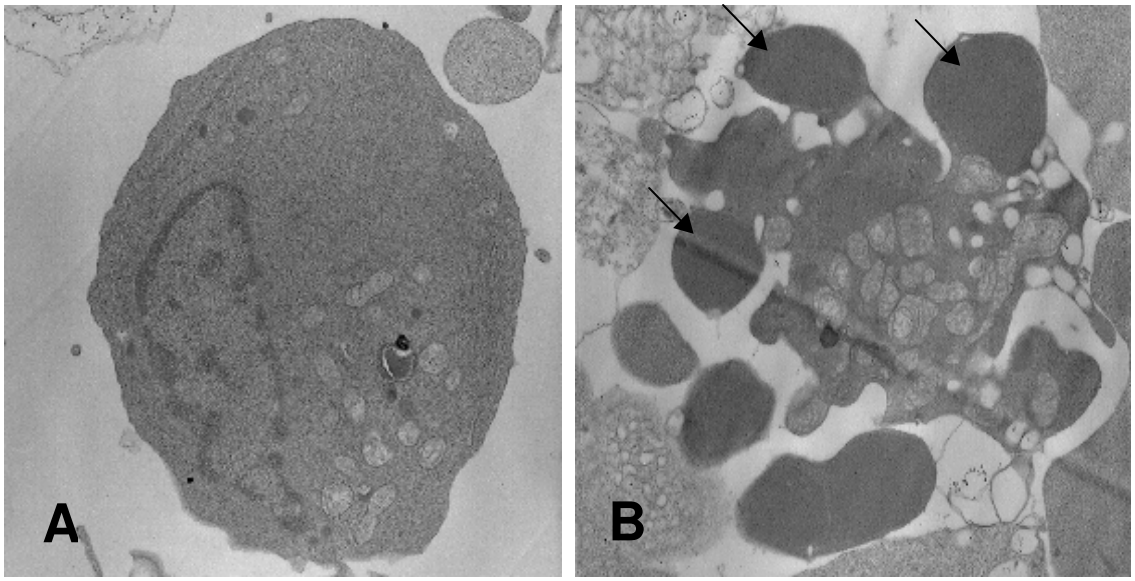

Figure 3-6. Transmission electron micrograph of RTS11 exposed to gliotoxin.

RTS11 cultures in serum free L-15 were exposed to 1.5 μM gliotoxin or carrier alone for 2h and then examined by transmission electron microscopy. Control cultures (**A**) demonstrated single, intact nuclei with uniform chromatin. Gliotoxin treated cultures (**B**) demonstrated cellular blebbing and nuclear fragmentation (indicated by arrows), both indicative of apoptosis. Both pictures were taken at 8900X magnification.

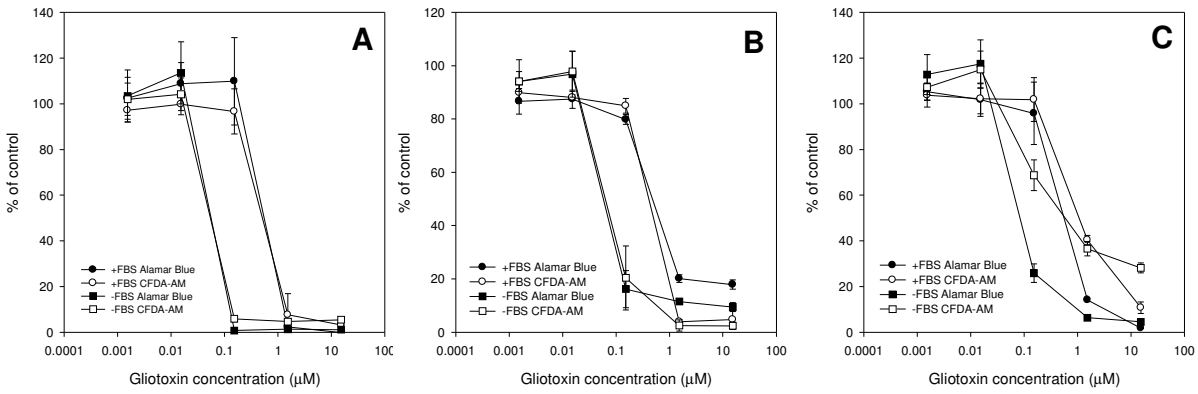

Figure 3-7. Effect of 24 h exposures to gliotoxin on the viability of three salmonid cells lines.

RTS11 (**A**), RTG-2 (**B**), CHSE-214 (**C**) were exposed to gliotoxin in L-15 with or without FBS. Cell viability was measured using two fluorescent indicator dyes, AB and CFDA-AM, and was expressed on the Y-axis as a % of control cultures treated with the carrier (EtOH) alone. The x-axis represents the concentration of gliotoxin in μM on a logarithmic scale. For each concentration, six wells were exposed and the mean and standard deviations are graphed. This graph is representative of three similar experiments (see Table 3-1).

exposed without FBS. In this case the EC_{50} with CHSE-214 was higher than those with RTS11 and RTG-2 (t-test, $p<0.05$;Table 3-1). As with 6 h exposures, 24 h EC_{50}s for RTS11 were higher in FBS than in L-15 alone (t-test, $p < 0.05$; Table 3-1).

3.3.5. Mode of cell death after 24 h gliotoxin exposures

After 24 h with 1.5 µM gliotoxin, all three cell lines showed hallmarks of apoptosis, nuclear fragmentation and DNA laddering. This was seen most definitively in cultures without FBS (Figure 3-8). H33258 staining was used to study nuclear fragmentation, and nuclear integrity was scored for cells that remained on the growth surface. For RTS11, all cells remained attached, but for the other two cell lines, some cells did detach. Among the attached cells, the % of fragmented nuclei was considerably higher in the 1.5 µM gliotoxin- treated cultures than in control cultures for all three cell lines (Figure 3-8 bottom panel). For RTG-2 and CHSE-214, the values underestimate the % of fragmented nuclei in the cultures as cells with fragmented nuclei were seen in the detached or floating population and were estimated at approximately 50% but could not be scored accurately. Floating cells were included along with attached cells in the extraction of gDNA from cultures. Electrophoresis of gDNA from all three types of cultures revealed clear ladders (Figure 3-8, top panel). This laddering also was seen in cultures with FBS (data not shown). However, in RTG-2 and CHSE-214 cultures with FBS, nearly all cells detached, and many fragmented nuclei could be seen in the floating cells but the % of fragmented nuclei could not scored easily.

After 24 h with 15 µM gliotoxin, RTS11 appeared to die differently than the other two cell lines. The majority of nuclei in RTS11 cultures with or without FBS were fragmented (Figure 3-5). DNA laddering also was evident, although the bands were not quite as prominent as at 1.5 µM gliotoxin (Figure 3-8). Very few fragmented nuclei were seen in RTG-2 and CHSE-214 cultures without FBS (Figure 3-5), and although not counted, very few were seen in cultures with FBS, which had nearly all detached cells. No DNA laddering was seen in either RTG-2 or CHSE-214 cultures at this concentration (Figure 3-8). These results suggested that although apoptosis continued in RTS11 cultures, an alternative death mode dominated in RTG-2 and CHSE-214 cultures treated with 15 µM gliotoxin.

FBS clearly modified the apoptotic response in RTS11, but this was less clear with the other two cell lines (Figure 3-5). Relative to control RTS11 cultures, gliotoxin caused a significant increase in the % of fragmented nuclei in cultures at 0.15 µM in L-15 alone but not in L-15 with FBS at 24 h (ANOVA, $p<0.05$). This also appeared to be the case for CHSE-214, but not with RTG-2, although for both cell lines the detachment of cells in L-15 and not in L-15 with

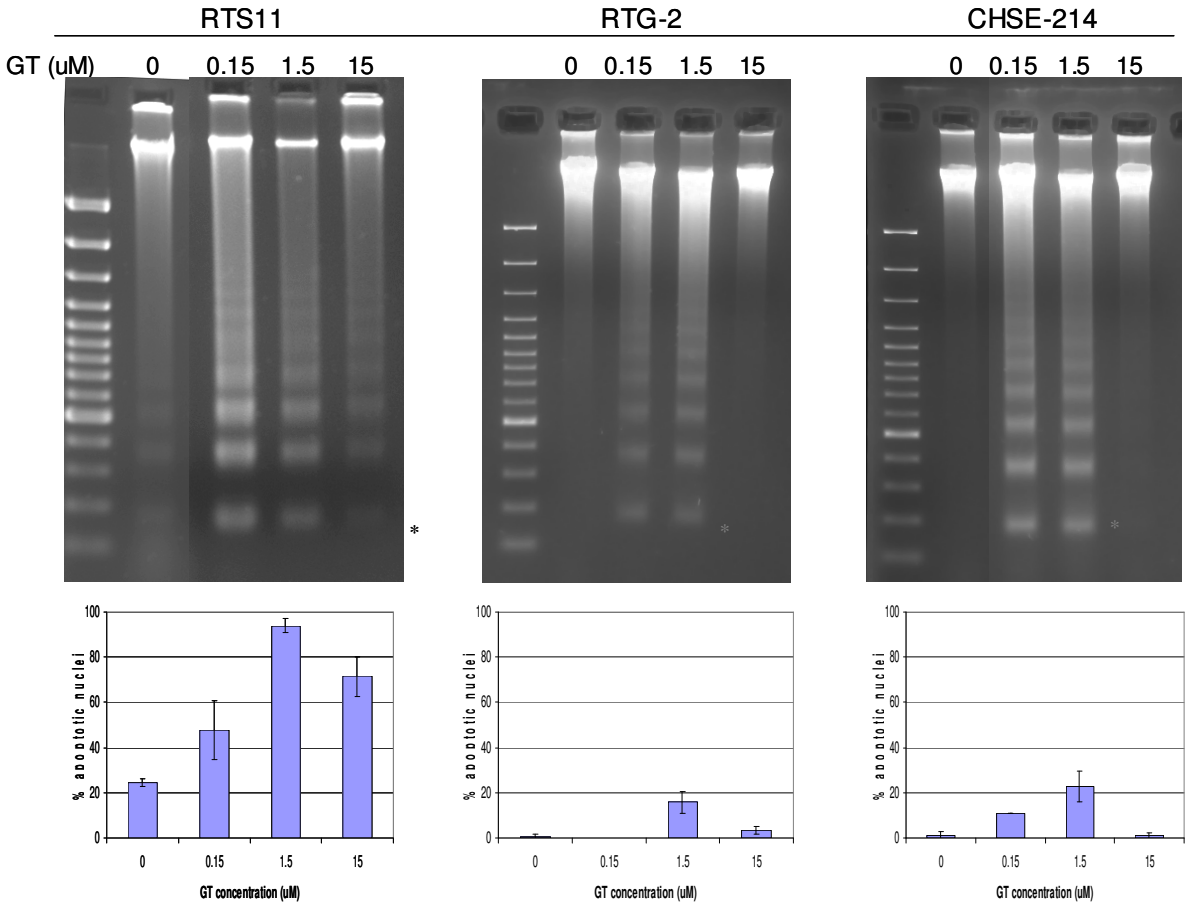

Figure 3-8. Effect of 24 h exposures to gliotoxin on the appearance of apoptotic hallmarks of three salmonid cells lines.

After being exposed to varying concentrations of gliotoxin in serum free L-15, RTS11, RTG-2 and CHSE-214 cultures were examined for two hallmarks of apoptosis, gDNA laddering and nuclear fragmentation. Genomic DNA laddering is shown in the top panels, with the position of the 180 bp fragment being identified by an asterisk. The percent of cells with fragmented nuclei was scored in H33258 stained cultures and are plotted in the bottom panels. In RTG-2 and CHSE-214 cultures at 0.15 and 1.5 µM gliotoxin, some cells detached and these were included in the DNA analysis, but only the attached cells were scored for nuclear fragmentation.

FBS complicated the issue (Figure 3-5). Finally, in serum-free L-15 without any gliotoxin treatment, RTS11 cultures, unlike RTG-2 and CHSE-214, had a significant number of apoptotic nuclei (Figure 3-5), although not always enough to yield 180 bp oligomers upon agarose electrophoresis of gDNA. The trigger for this apoptosis is under investigation.

3.4. DISCUSSION

As judged by several endpoints, gliotoxin was cytotoxic to the salmonid cell lines, RTS11 (monocyte/macrophage), RTG-2 (fibroblast) and CHSE-214 (epithelial), with cytotoxicity varying somewhat between lines and being modulated by fetal bovine serum (FBS). One of the most sensitive measures was an impairment of metabolism as measured by the reduction of alamar Blue (AB) (resazurin). EC_{50} values after 6 h but not 24h exposures were lower than the measure of plasma membrane integrity, the CFDA-AM assay. This could be due to gliotoxin's early disruption of mitochondrial activity, as the toxin has been shown to target mitochondria in mammalian cells (Kweon et al., 2003). For RTS11, the EC_{50}s from both viability assays were significantly higher when FBS was present. This suggests that FBS protected cells either directly by encouraging cell health or indirectly by binding gliotoxin making less available to exert toxicity. Yet, FBS had little or no impact on these viability endpoints in RTG-2 and CHSE-214 cultures. Conversely, for RTG-2 and CHSE-214 but not RTS11 gliotoxin caused cell detachment after 24 h, and this was most obvious at intermediate concentrations (0. 15 and 1.5 μM) in cultures without FBS and higher concentrations (1.5 and 15 μM) in cultures with FBS. Previously gliotoxin was observed to detach adherent mammalian cells in primary cultures and cell lines (Jordan and Pedersen, 1986; Piva, 1994). Gliotoxin has been reported to inhibit macrophages from attaching to plastic surfaces (Waring et al., 1988) but macrophage detachment appears not to have been described. As judged by the EC_{50}s from the AB and CFDA-AM assays, cell viability continued to decline between 6 and 24 h. By the same criteria, little difference was found between the cell lines, suggesting a similar sensitivity to the overall killing by gliotoxin. Finally, as discussed below, in cultures that by the above endpoints had lost viability, the features of apoptosis were observed after 24 h in some cases, and absent in other situations, suggesting that alternative modes of killing also were possible. This was assumed to be necrosis as this is the most common alternative (Edinger and Thompson, 2004). As well as being a response to extreme conditions, necrosis can be considered a normal physiological and programmed event under some circumstances and after some stimuli (Proskuryakov et al., 2003).

The capacity to undergo apoptosis was demonstrated in the three salmonid cell lines. Nuclear fragmentation and DNA laddering are characteristic features of apoptosis (Loo and Rillema, 1998), and these hallmarks were observed consistently in cultures at some time during gliotoxin treatment. In contrast to RTS11, demonstrating this process in RTG-2 and CHSE-214 was surprisingly difficult, requiring a longer exposure time, occurring under specific culture conditions, and being generally less pronounced. Previously, RTS11, but not RTG-2 and CHSE-

214, was shown to die by apoptosis in response to transcriptional- and translational- inhibitors and double-stranded RNA (dsRNA) (DeWitte-Orr et al., 2005a). In CHSE-214, infectious pancreatic necrosis virus (IPNV) induced apoptosis (Hong et al., 1998) and at least some of the apoptotic machinery, caspase 6, has been demonstrated (Miwa et al., 2002). Recently butylated hydroxyanisole was shown to cause necrosis and apoptosis in RTG-2 cultures, with the latter demonstrated by the TUNEL assay (Jos et al., 2005). Thus, although capable of apoptosis, the cell lines would appear to have some intrinsic differences either in their competence for this death mode or in how they respond to the inducing agents that have been studied to date. Overall, demonstrating the capacity of these salmonid cell lines to undergo apoptosis should improve their utility in toxicology and ecotoxicology, helping in the choice of appropriate cell lines and culture conditions for demonstrating this mode of cell death.

In this, the first systematic investigation of gliotoxin's cytotoxicity to fish cells, the responses of salmonid cell lines were found to be similar with those of mammalian cell lines. Gliotoxin has been shown to be toxic to mammalian cells from different tissues (Beaver and Waring, 1994), and this seemed true for salmonid cells. As well as the cell lines of the current study, gliotoxin was cytotoxic to the rainbow trout gill epithelial cell line, RTgill-W1 (Dayeh et al., 2005). For mammalian cells, cytotoxicity usually has been observed in the 0.1- 3 μM range (Beaver and Waring, 1994), which encompassed the EC_{50} values for the salmonid cell lines as determined by the viability assays used. For gliotoxin-induced apoptosis of mammalian cells, immune cells have appeared more sensitive than other cell types (Braithwaite et al., 1987), which was echoed in the salmonid cell lines by the monocyte/macrophage cell line, RTS11, being more susceptible to this form of death.

At least under some conditions, gliotoxin clearly killed the fibroblast and epithelial cell lines by an alternative mode to apoptosis, likely necrosis. RTG-2 and CHSE-214 exposed to 15 μM gliotoxin in the absence of FBS for 24 h were clearly dead as judged by viability assays, and confirmed by their profoundly altered morphological appearance. Hallmarks of apoptosis were absent in these cultures. As no apoptotic cells were even seen at 6 h, the absence of apoptotic cells at 24 h suggests that all RTG-2 and CHSE-214 cells died by necrosis early in the exposure, which is often the case with extreme stimuli (Proskuryakov et al., 2003). Specifically, the mode of death for many mammalian cells has been found to be *via* necrosis instead of apoptosis at concentrations above 10 μM (Hurne et al., 2002), which also appears to be the case under these conditions for RTG-2 and CHSE-214. By contrast, for RTS11 with or without FBS, apoptotic cells were seen at 6 h and even more at 24 h, with over 80 % being apoptotic. This suggests that

at the highest gliotoxin concentration most RTS11 died by apoptosis, and if any did die by necrosis, the numbers were small.

The appearance of apoptotic cells in gliotoxin-treated CHSE-214 and RTG-2 cultures required 24 h exposures and correlated with detachment of cells from the plastic growth surface. On the basis of individual cells, the correlation between cells being detached and undergoing apoptosis was not precise. Some detached cells did not have fragmented nuclei, and some attached cells were apoptotic. However, with one exception, which is discussed in the following paragraph, the correlation held at the culture level. Hallmarks of apoptosis were always seen in cultures in which at least some of the cells had detached. In those cultures in which both floating and adherent cells were present, the proportion of apoptotic cells appeared higher in the detached population. The reasons behind the concurrence of detachment and apoptosis in RTG-2 and CHSE-214 cultures can only be speculated on at this time. Apoptotic cells could be preferentially detached. Alternatively, as these two cell lines appear to be able to die by either necrosis or apoptosis in response to gliotoxin, and do so in the same culture, detachment could be a trigger to favor the apoptotic over the necrotic pathway.

Treatment of RTG-2 and CHSE-214 in FBS to 15 μM gliotoxin was the one exception to the concurrence of detachment and apoptosis. Under these conditions the cells had all detached but apoptotic hallmarks were absent or much less evident. With 15 μM gliotoxin most cells appeared to have died by necrosis with or without FBS, but when FBS was present, the dead cells detached. The detachment might have been by a different mechanism than that which occurred at lower gliotoxin concentrations. Without FBS, nearly all dead cells remained attached. The cells likely were attached slightly differently to the plastic surface with FBS than without FBS, and this could account for detachment occurring in the first case and not in the second.

The difference between RTS11 versus CHSE-214 and RTG-2 could be in how adherence is regulated and how this in turn regulates apoptosis. Although RTS11 cells grow loosely suspended in cultures with FBS and become more strongly adherent without FBS(Ganassin et al., 2000; Pagniello et al., 2002; Ganassin and Bols, 1998), gliotoxin did not cause a notable detachment under either condition and the level of apoptotic cells was highest without FBS, when the cells were most adherent. By contrast, the other two cell lines adhere strongly to the growth surface, and apoptosis was most evident only upon detachment of the cells. Integrins, which mediate cell adhesion, are usually restricted to certain cell types (Hynes, 1992), and might be expected to differ between RTS11 and the adherent cell lines. Fibronectin (Fn), which is a ligand for several integrins, has been shown to prevent the apoptosis of human cells in culture by

integrin-dependent mechanisms (Scott et al., 1997). Although knowledge of salmonid integrins is limited, RTG-2 express integrins and attach to Fn (Lee and Bols, 1991; Reinhart and Lee, 2002).

The apparent preferential death by necrosis for adherent epithelial cells and by apoptosis for macrophages and for detached epithelial and fibroblast cells could be a beneficial host response to gliotoxin-producing fungi infection, which presumably begins at an intact adherent layer of epithelial cells. The rapid killing of a few epithelial cells by necrosis would release cytosolic components that could initiate ultimately an adaptive immune response but more immediately an inflammatory response, which would include the attraction of the macrophages to assist in killing the fungus and regulating the resolution of the inflammation. Later during the fungal infection and associated inflammatory response, epithelial and fibroblasts might detach from the extracellular matrix and having these detached cells and the macrophages die by apoptosis would allow them to be safely removed by other macrophages and prevent further provocation of the inflammatory response.

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Chapter 4

A comparison of antiviral gene induction, Mx and vig-1, between rainbow trout fibroblast and moncyte/macrophage cell lines by dsRNA and chum salmon reovirus – a possible role for PKR

ABSTRACT

The expression of potential antiviral genes, *Mx* and *vig-1*, was studied in two rainbow trout cell lines: monocyte/macrophage RTS11 and fibroblast-like RTG-2. By RT-PCR for the four genes, *Mx1*, *Mx2*, *Mx3* and *vig-1*, and by Western blotting for Mx, expression in control cultures was not common in RTG-2, but in RTS11 Mx protein was detected and occasionally Mx transcripts were seen. In both cell lines, transcripts for all four genes and Mx protein were induced by a dsRNA, poly inosinic:cytidylic acid (poly IC), by Chum salmon reovirus (CSV), and by medium conditioned by cells previously exposed to poly IC or CSV and assumed to contain type I interferon (IFN), thus referred to as interferon- containing medium (ICCM). In both cell lines, poly IC or CSV with cycloheximide continued to induce Mx transcripts, suggesting that a direct induction mechanism, independent of IFN, was also possible. For CSV, ribavirin blocked induction in RTS11, but not in RTG-2, suggesting viral RNA synthesis was required for induction only in RTS11. Poly IC activated dsRNA-dependent protein kinase (PKR) activity in both cell lines yet PKR inhibitors blocked induction of the four genes by poly IC, CSV, and ICCM in RTS11 only. These results suggest that PKR is not involved in RTG-2 induced *Mx* and *vig-1* expression, thus highlighting differences in antiviral gene induction between the two cell lines.

4.1. INTRODUCTION

Manipulating the antiviral mechanisms of fish is of interest in aquaculture as a possible means of enhancing protection against viral diseases but is limited by the relative lack of basic knowledge about such mechanisms and how they are regulated. One potential target for manipulation is the Mx proteins (Lockhart et al., 2004; Salinas et al., 2004; Leong et al., 1998). Mx proteins were originally discovered in influenza-resistant laboratory mice (Lindenmann, 1962), and have since been identified in many vertebrate species including fish (Lee and Vidal, 2002). They are dynamin-related members of the large GTPase super-family (Lee and Vidal, 2002). Most animals have multiple Mx isoforms, and rainbow trout express three Mx proteins (Trobridge et al., 1997b). Recently an antiviral role for Mx in fish has been described, with Atlantic salmon Mx1 inhibiting infectious pancreatic necrosis virus replication (IPNV) (Larsen et al., 2004) and infectious salmon anemia virus (ISAV) (Kibenge et al., 2005b). Virus induced gene–1 (vig-1) was first identified in viral hemorrhagic septicemia virus (VHSV) exposed rainbow trout leukocytes (Boudinot et al., 1999). Subsequently, viperin and cig5, which share sequence similarity with vig-1, have been identified in humans and mice and possible antiviral activity has been demonstrated (Boudinot et al., 2000).

A variety of agents have been explored both *in vivo* and *in vitro* for their capacity to enhance Mx expression, one common stimulant is the synthetic double-stranded RNA (dsRNA), polyinosinic: polycytidylic acid (poly IC) (Eaton, 1990; Lockhart et al., 2004). Poly IC has also been shown to induce vig-1 expression (Alonso and Leong, 2002); however, concurrent induction of vig-1 and Mx has yet to be demonstrated. Another potential inducer of antiviral genes that has yet to be examined is Chum salmon reovirus (CSV), which is an aquareovirus (AqVR) (Essbauer and Ahne, 2001). AqRV are non-enveloped with an icosohedral, double capsid and an eleven segment dsRNA genome (Winton et al., 1981). CSV was isolated from apparently healthy fish and caused little or no mortality in several salmon species (Winton et al., 1981). A subsequent study showed that CSV was able to stimulate host defenses in rainbow trout and protect live fish against infections hematopoietic necrosis virus (IHNV), which is a rhabdovirus and a serious viral pathogen for rainbow trout farms (LaPatra et al., 1995). Although the mechanism was not investigated, induction of antiviral gene (s) is a possibility.

Central to the regulation of antiviral defenses in mammals, and presumably fish, is a network of interferons, coordinated by interacting signal transduction pathways. Type I interferons (IFNs) are cytokines induced by viruses that act in an autocrine and paracrine fashion, through their cognate receptors, to induce expression of a battery of genes (interferon stimulated genes, ISGs) that collectively bestow an antiviral state. Interferons can affect the expression of over 1000

different genes in mammalian cells (Der et al., 1998). One of the IFN-inducible proteins in mammals is the dsRNA-dependent protein kinase (PKR), which is a serine/threonine kinase (Sen, 2000). PKR, which is synthesized inactive, is activated by dsRNA; however, PKR activity is not limited to antiviral mechanisms and has increasingly been found to be a regulator of diverse cellular responses to stress (Peel, 2004). In fish only a handful of ISGs have been identified, including *Mx* and *vig-1*. A PKR-like gene has been recently identified in the crucian carp (Hu et al., 2004a), and zebrafish (Rothenburg et al., 2005) and PKR activity has been demonstrated in rainbow trout (Garner et al., 2003). Poly IC has been shown to stimulate another type of antiviral defense, apoptosis, in the rainbow trout macrophage-like cell line, RTS11, which appeared to be mediated by PKR (DeWitte-Orr et al., 2005). By contrast, a rainbow trout fibroblast cell line, RTG-2, was unresponsive to dsRNA-killing (DeWitte-Orr et al., 2005).

In this report the capacity of poly IC and CSV to induce *Mx1*, *Mx2*, *Mx3*, and *vig-1* has been compared in RTS11 and RTG-2 and possible IFN dependent and independent pathways have been investigated. As well, the possible role of PKR in these induction pathways has been explored.

4.2. MATERIALS AND METHODS

4.2.1. Cell Culture

Two cell lines were used in this study, a fibroblast gonadal cell line, RTG-2, and a macrophage-like cell line, RTS11, both derived from rainbow trout. RTG-2 was obtained from the American Type Culture Collection (ATCC), whereas RTS11 was developed in this laboratory (Ganassin and Bols, 1998). The routine growth of these cells lines has been described in detail previously (Bols and Lee, 1994; DeWitte-Orr et al., 2005). Briefly, cultures were maintained at 18-21 °C in Leibovitz's L-15 supplemented with fetal bovine serum (FBS) and penicillin G/streptomycin sulfate. These items were purchased from Sigma (St. Louis, MO). RTG-2 cultures were grown in 75 cm^2 plastic tissue culture flasks while RTS11 cultures were grown in 25 cm^2 plastic tissue culture flasks, both flasks were purchased from Nunc (Roskilde, Denmark).

4.2.2. Virus propagation and infection

Chum salmon reovirus (CSV) was obtained from ATCC and routinely propagated on monolayers of a chum salmon embryonic cell line, CHSE-214. CSV containing media (CCM) was collected 7d post-infection (pi), passed through a 0.2 μm filter, and kept frozen at -80°C until used. Virus titration was performed on monolayers of CHSE-214 cells grown in a 96-well plate (Falcon/Becton-Dickenson, Franklin Lakes, NJ). Viral suspensions were diluted from 10^{-1} to 10^{-6} and 6 wells were inoculated with 200 μL of each dilution. Cultures were incubated at 21 °C for 3 days. Following this period, the cell monolayers were scored for the appearance of cytopathic effects and the final titre, expressed as $TCID_{50}$ /mL, was estimated using the Karber method (Karber, 1931).

4.2.3. Cell treatments

RTG-2, were seeded at $2.3x10^6$ cells per 25cm^2 tissue culture flasks, while RTS11 were plated at $10x10^6$ cells per 12.5cm^2 tissue culture flask. All cells were plated in L-15 media without FBS supplementation. Cells were allowed to attach overnight at normal growing temperatures before being treated.

Interferon containing conditioned media (ICCM)

RTS11 and RTG-2 were treated with a stimulant, either 50 μg/mL poly IC (Sigma) or chum salmon reovirus (CSV; $10^{4.3}$ $TCID_{50}$/mL) for 24h. After which, the media was removed and the cells were washed two times with Dulbecco's phosphate buffered saline (D-PBS; Sigma) and fresh media was added. As RTS11 cultures are semi-adherent, stimulant exposures were

terminated slightly differently. The cells were collected, centrifuged and washed twice with PBS in a 15mL polypropylene centrifuge tube (Falcon/Becton-Dickenson, Franklin Lakes, NJ), and plated in new tissue culture treated flasks with fresh media. The cells were then allowed to condition this media for 24h before it was collected, filtered (0.2μm filter), and immediately added, undiluted, to different cell cultures for measuring gene expression. Unless otherwise indicated, RTS11 ICCM was applied to RTS11 cultures, and RTG-2 ICCM was applied to RTG-2 cultures. In this chapter, ICCM denotes interferon containing conditioned media from poly IC stimulated cells, while CSV-ICCM denotes ICCM from CSV stimulated cells. Control cultures for these experiments received conditioned media from untreated cells and are indicated as CM-control.

Stimulant study

RTS11 and RTG-2 cultures were treated with 50 μg/mL poly IC, CSV ($10^{4.3}$ $TCID_{50}$/mL), undiluted ICCM or CSV-ICCM. Cells were incubated for 24h at 18°C.

Inhibitor study

RTS11 were pretreated with inhibitors for 30 minutes prior to treatment with a stimulant. Inhibitor treatments included two PKR inhibitors, 1.5 mM 2-aminopurine (2-AP), and a second commercially available oxindole functionalized with an imidazole, referred to as compound 16 (Jammi et al., 2003)(0.5 μg/mL), a translation inhibitor, cycloheximide (0.1μg/mL) and an antiviral compound, ribavirin (100 μg/mL). All inhibitors were purchased from Sigma with the exception of compound 16, which was purchased from Calbiochem (La Jolla, CA). Following the 30 minute pretreatment, cells were treated with poly IC, CSV or ICCM as described in the stimulant study section.

4.2.4. RT-PCR

RNA extraction

Cells were collected at specific time points, pelleted and washed with D-PBS. RNA was extracted using GenElute mammalian total RNA miniprep kit (Sigma). RNA was eluted using nuclease free water (Bioshop, Burlington, ON).

cDNA synthesis

2 μg RNA was treated with 1U RNnase-free, DNase I (Roche, Laval, Qc) for 30 minutes at 37°C and 5 minutes at 75°C to remove any contaminating genomic DNA from the samples.

These samples were then used to obtain cDNA as follows. 1µL of 0.5µg/mL oligo-$(dT)_{23}$ anchored primer (Sigma) was added to each of the RNA samples, the samples were heated to 70°C for 10 minutes and immediately put on ice. Afterward, 4µL 5X buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, Invitrogen, Burlington, ON), 1µL 10mM deoxynucleotide triphosphate (dNTP, Sigma) mix, 2µL 0.1 M dithiothreitol (DTT, Invitrogen) and 80 U Superscript II reverse transcriptase (Invitrogen) were added to each sample. The reaction was allowed to sit at room temperature for 10 minutes prior to 42°C for 50 minutes and 95°C for 5 minutes. The resulting cDNA was diluted 1:20 in nuclease free water and stored at –80°C.

PCR reactions

All PCR reactions contained: 0.5 µL 10 mM dNTP mix (Sigma), 1.25 U Taq polymerase (Sigma), 1.5 mM $MgCl_2$ (Sigma), 2.5 µL 10X reaction buffer (500mM KCl, 100mM Tris·HCl, pH 8.3, Sigma), 1.25 µL 10µM forward and reverse primers, 2.5µL diluted cDNA and nuclease free water to a 25 µL total volume. All PCR reactions were setup on ice. The primer sequence, cycle number, and annealing temperature for each primer set are listed in Table 4-1. The PCR reactions were run using a Mastercycler personal thermocycler (Eppendorf), all samples were run at least twice with two independent sets of cDNA to verify results. Cycle conditions were as follows: 95°C for 2 minutes, a set number of cycles with 95°C 45s, primer-specific annealing temperature for 45s, 72°C for 45s followed by 72°C for 10 minutes. PCR products (10 µL) were visualized on a 1.5% agarose gel with 15 min post-stain in 0.5µg/mL ethidium bromide (EtBr) and 15 min destain in MilliQ water followed by visualization under UV transillumination.

Amplified primer sequences were cloned into pGEM-Teasy (Promega, Madison, WI) and subsequently sequenced to confirm primer specificity, especially between *Mx1*, *Mx2* and *Mx3*. All primers were found to amplify the gene of interest specifically.

Table 4-1. Summary of primers used in study, including PCR product size and number of cycles used.

Gene accession numbers are listed in brackets.

Gene	Primers	PCR product size (bp)	Annealing temperature	Cycle #	Primer Reference
β-actin (AJ438158)	F 5' ATCGTGGGGCGCCCCAGGCACC 3' R 5' CTCCTTAATGTCACGCACGATTTC 3'	514	53°C	30	(Brubacher et al., 2000)
Mx1 (U30253)	F 5' ATGCCACCCTACAGGAGATGAT 3' R 5' AAAAAGGATAACAAAGGACT 3'	452	53°C	30	(Collet and Secombes, 2001)
Mx2 (U47945)	F 5' CTTGGTAGACAAAGGCACAGAGGA 3' R 5' AAGTTCTTTCCAGAGCGATCCA 3'	500	65°C	27	unpublished
Mx3 (U47946)	F 5' ATGCCACCCTACAGGAGATGAT 3' R 5' CCACAGTGTACATTTAGTTG 3'	380	53°C	30	(Collet and Secombes, 2002)
Vig-1 (AF076620)	F 5' CAGTTCAGTGGCTTTGACGA 3' R 5' ACAAACTCCTCAAGGTATGG 3'	232	65°C	27	(Boudinot et al., 1999)

4.2.5. Western blot analysis

RTS11 cultures were seeded at $1.0x10^7$ cells in 12.5 cm^2 flasks while RTG-2 cultures were seeded at $2.3x10^6$ cells in 25cm^2 flasks, cultures were allowed to reattach overnight at 18°C. Cells were treated as described above (sections 4.2.3) and collected at the completion of the exposures. Cells were washed twice in ice cold PBS and lysed on ice for 30 minutes in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris, pH 8.0) with protease inhibitor cocktail (Sigma) added. Collection of protein for the eIF2α phosphorylation study was more exhaustive, as previously described (Savinova and Jagus, 1997). Briefly, cells were kept on ice while being collected, and only three samples were collected at once to limit time between collection and lysis. Cells were washed in ice cold PBS+100mM NaF and 5mM EDTA, and were lysed using a modified RIPA buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 50mM Tris, 100 mM NaF, 5mM EDTA, pH 8.0). For the eIF2α phosphorylation study, all samples were lysed in the modified RIPA, scraped, collected and prepared for centrifugation within 5 minutes. The lysate was centrifuged for 5 minutes at 10 000 xg and stored at -80°C until used. In less stringent situations, the cells were lysed for 30 minutes and centrifuged at 10 000 xg for 15 minutes. In both cases, the protein concentration in the cell extract was quantified using the Bradford method. Equal amounts of protein (20μg) were boiled for 5 minutes in Laemmli buffer (135mM Tris, 4% (w/v) SDS, 0.06% (w/v) bromophenol blue, 20% glycerol, 2 % (w/v) 2-Mercaptoethanol), put immediately on ice and added to each lane of a SDS-15% polyacrylamide gel, with the exception of the eIF2α phosphorylation study, where samples were run on a SDS-10% polyacrylamide gel. Electrophoresis was performed using a Bio-Rad mini-PROTEAN II

electrophoresis cell. SeeBlue pre-stained protein standard (Invitrogen) was included with each gel for protein size estimation. Proteins were electro-transferred to nitrocellulose membrane (Bio-Rad) overnight using the Bio-Rad mini-Trans Blot cell (60mA). Equal loading of protein samples was confirmed by staining of total protein with 0.1% Ponceau S (in 5% w/v acetic acid). The stained blots were scanned and de-stained using dH_2O. Membranes were then blocked in 5% (w/v) nonfat dried milk in T-TBS (10mM Tris-HCl, 100mM NaCl, 0.1% (v/v) Tween 20) for 1h at room temperature (RT). The primary antibody was diluted in blocking solution: polyclonal rabbit anti-rainbow trout Mx (Trobridge et al., 1997) was diluted 1:2000, monoclonal mouse anti-human eIF2α (Biosource, Camarillo, CA) was diluted 1:1000 and the polyclonal rabbit anti-human phosphorylated eIF2α (Biosource) was diluted 1:1000. The membranes were incubated with the primary antibody for 1h at RT. Membranes were then washed three times in T-TBS and incubated with the secondary antibody; horseradish-peroxidase-conjugated goat anti-rabbit or mouse IgG (H+L) (BioRad), as appropriate, and visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Little Chalfont, England). Each blot was performed at least twice with independent samples.

4.3. RESULTS

4.3.1. Constitutive expression

RT-PCR was used to study the constitutive expression of *vig-1* and *Mx1*, *Mx2* and *Mx3* transcripts in two rainbow trout cell lines, the macrophage-like RTS11, and the fibroblast-like RTG-2. Constitutive expression of *Mx* and *vig-1* transcripts was seen occasionally in RTS11 cultures, but rarely or not at all in RTG-2 cultures. Western blotting with antibodies against rainbow trout Mx was used to detect constitutive expression of the Mx protein (Figure 4-7). A broad band at 70 kDa, which sometimes appeared as a doublet, was repeatedly seen in untreated RTS11 cultures but was rare in RTG-2 cultures.

4.3.2. *Mx* and *vig-1* transcript induction by dsRNA

Polyinosinic-polycytidylic acid (poly IC) exposures of 24 h induced *Mx1*, *Mx2*, *Mx3* and *vig-1* in both RTS11 and RTG-2 (Figure 4-1). Gene expression seemed faster in RTS11, where faint bands of *Mx2*, *Mx3* and *vig-1* could be detected after 4h exposures while only *Mx2* transcript could be detected in RTG-2.

4.3.3. *Mx* and *vig-1* transcript induction by interferon-containing conditioned medium (ICCM)

ICCM, medium conditioned by RTS11 or RTG-2 previously exposed to poly IC, was applied to RTS11 and RTG-2 cultures and *Mx* and *vig-1* expression was monitored at the transcript level. ICCM induced strong *Mx* and *vig-1* expression in RTS11 as early as 4h at the transcript level and could still be detected at 24h as well. RTG-2 control cultures receiving control CM demonstrated low levels of *Mx2* and *Mx3* transcripts, these levels were not enhanced in cultures treated with ICCM (Figure 4-2).

4.3.4. Effect of PKR inhibitors on induction by dsRNA or ICCM

The involvement of PKR in the induction of *Mx* and *vig-1* in RTS11 was investigated using two inhibitors, 2-aminopurine (2-AP) and compound 16. In RTS11, induction of *Mx* and *vig-1* expression by poly IC and was completely blocked by both PKR inhibitors (Figure 4-3B). ICCM induced *Mx* and *vig-1* was also inhibited by compound 16 (Figure 4-2), following 24h exposures to the stimuli. Following 4h exposures to poly IC, compound 16 also blocked *Mx* and *vig-1* expression in RTS11 (data not shown). Also, 4h and 24h exposures to poly IC showed PKR activity as measured by eIF2α phosphorylation using western blot analysis (Figure 4-3A). These results contrast with RTG-2, where poly IC did

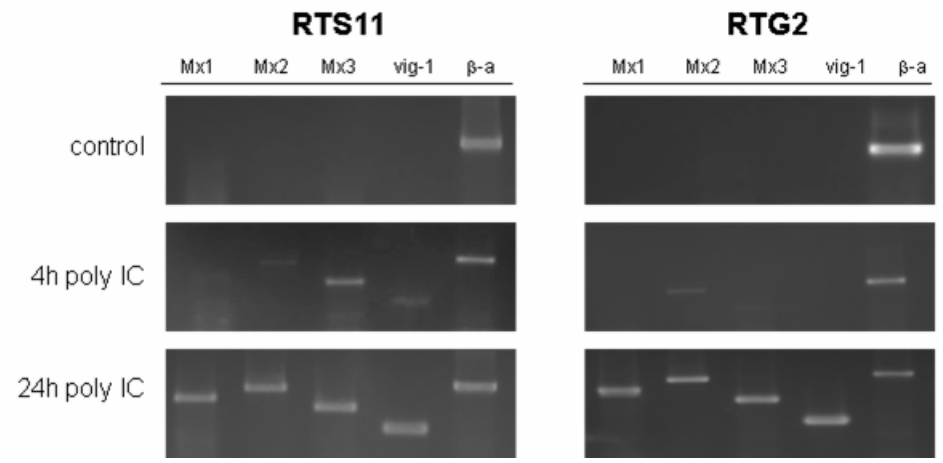

Figure 4-1. The effect of poly IC exposure on Mx and vig-1 expression in RTS11 and RTG-2.

RTS11 and RTG-2 cultures were exposed to 50 μg/mL poly IC for 4 or 24h. Cells were then collected and RT-PCR performed to measure *Mx* and *vig-1* expression. Individual bands represent expression of *Mx1*, *Mx2*, *Mx3, vig-1* and *β-actin* (β –a) at the transcript level as indicated in the headings at the top of the figure. Subsequent figures follow a similar gel layout.

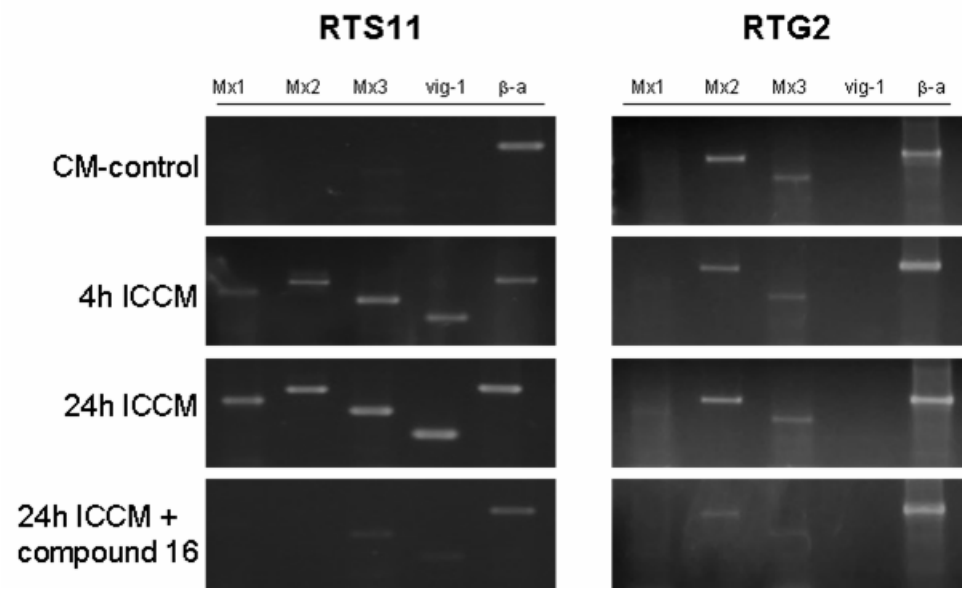

Figure 4-2. The effect of interferon containing conditioned (ICCM) media and a role for PKR in Mx and vig-1 expression in RTS11 and RTG-2.

RTS11 and RTG-2 were exposed to ICCM, from cultures previously treated with poly IC and consequently allowed to condition media, for 4 and 24h, with or without co-treatment with compound 16 (0.5 μg/mL), a PKR inhibitor. After these treatments, gene expression was measured using RT-PCR.

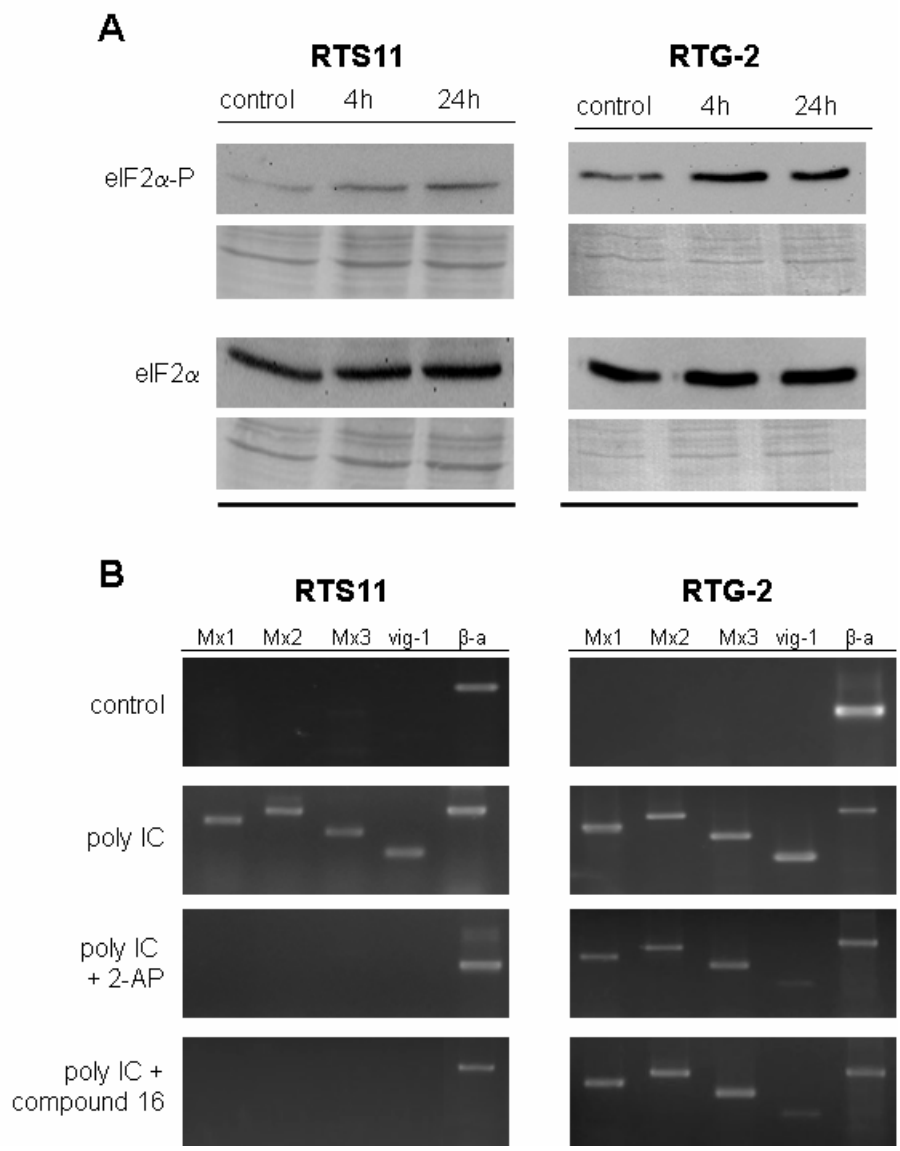

Figure 4-3. A role for the dsRNA dependent protein kinase, PKR, in poly IC-induced gene expression.

A Levels of phosphorylated eIF2α (eIF2α-P) and native eIF2α were measured in RTS11 and RTG-2 cultures exposed to poly IC for 4 or 24h using western blot analysis. **B** *Mx* and *vig-1* transcript levels were measured by RT-PCR following exposures to poly IC in combination with PKR inhibitors, 2-AP (1.5mM) and compound 16 (0.5 μg/mL), for 24 h.

induce PKR activity (Figure 4-3A), but Mx and vig-1 gene expression was not inhibited by either PKR inhibitor after 24h (Figure 4-3B). Compound 16 may have slightly inhibited the low levels of Mx2 and Mx3 gene expression in RTG-2 during ICCM treatment (Figure 4-2).

4.3.5. Effect of cycloheximide on induction by dsRNA

Evidence for a direct pathway to *Mx* and *vig-1* induction that does not require *de novo* protein synthesis was pursued using poly IC and cycloheximide (CHX), a translation inhibitor. RTS11 and RTG-2 cultures treated with poly IC and CHX together for 24h expressed *Mx* and *vig-1* transcripts similarly to cultures treated with poly IC alone (Figure 4-4).

4.3.6. *Mx* and *vig-1* transcript induction by chum salmon reovirus (CSV) and CSV-ICCM

RTS11 and RTG-2 cultures were exposed to CSV ($10^{4.3}$ $TCID_{50}$/mL) for 24h in combination with three different inhibitors: ribavirin, an inhibitor of viral transcription, cycloheximide, an inhibitor of translation, and compound 16, an inhibitor of PKR (Figure 4-5). CSV induced *Mx1*, *Mx2*, *Mx3* and *vig-1* transcript expression in both RTS11 and RTG-2, although *vig-1* transcript levels were consistently low in RTG-2. Ribavirin and compound 16 almost completely blocked *Mx* and *vig-1* transcript expression in RTS11, while cycloheximide only slightly affected transcript levels. Gene expression patterns were different in RTG-2, where ribavirin did not affect *Mx* and *vig-1* transcript levels, compound 16 did not affect *Mx* but blocked *vig-1*, and cycloheximide almost completely blocked *Mx* and *vig-1* transcript induction. Poly IC-induced *Mx* and *vig-1* transcript expression was not affected by ribavirin in either cell line (data not shown).

CSV-ICCM, from RTS11 and RTG-2 cultures previously exposed to CSV, induced *Mx* and *vig-1* expression in both RTS11 and RTG-2 (Figure 4-6). Compound 16 did not block transcript induction in RTG-2, but slightly affected expression in RTS11.

4.3.7. Mx expression at the protein level

Western blotting was used to detect Mx protein in RTS11 and RTG-2 cultures following exposures to poly IC and CSV (Figure 4-7). An enhancement of Mx protein levels was seen in both cell lines after 24 h. Interestingly, Mx protein levels decreased over time in RTS11, but stayed at high levels in RTG-2.

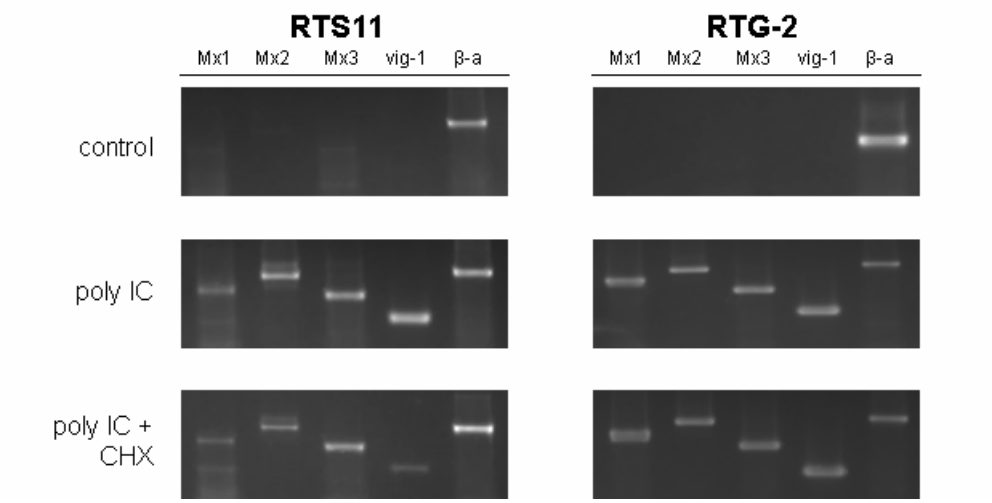

Figure 4-4. Effect of translation inhibitor, cycloheximide, on poly IC induced *Mx* and *vig-1* expression.

RTS11 and RTG-2 cultures were exposed to 50 μg/mL poly IC with or without co-treatment with 0.1μg/mL cycloheximide (CHX) for 24h. *Mx* and *vig-1* transcript expression was measured using RT-PCR.

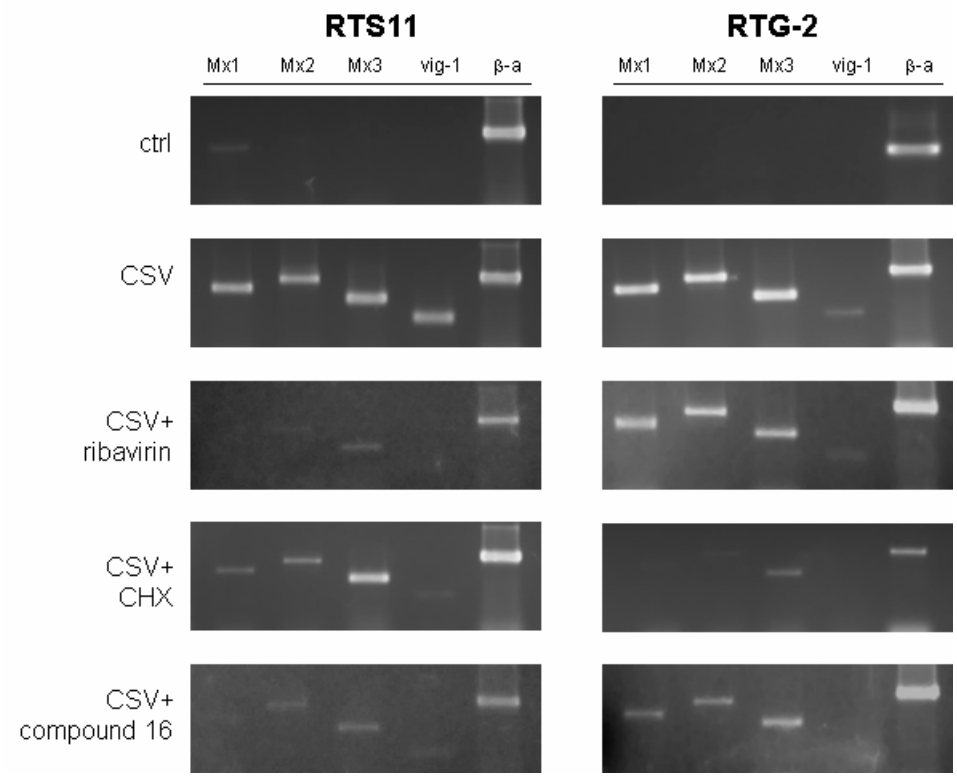

Figure 4-5. Effect of chum salmon reovirus, CSV, in combination with inhibitors of viral replication, translation and PKR, on *Mx* and *vig-1* expression.

RTS11 and RTG-2 cells were exposed to CSV ($10^{4.3}$ $TCID_{50}$/mL) with or without co-treatments with 100 µg/mL ribavirin, 0.1 µg/mL cycloheximide (CHX), or 0.5 µg/mL compound 16 for 24h. *Mx* and *vig-1* transcript levels were measured using RT-PCR.

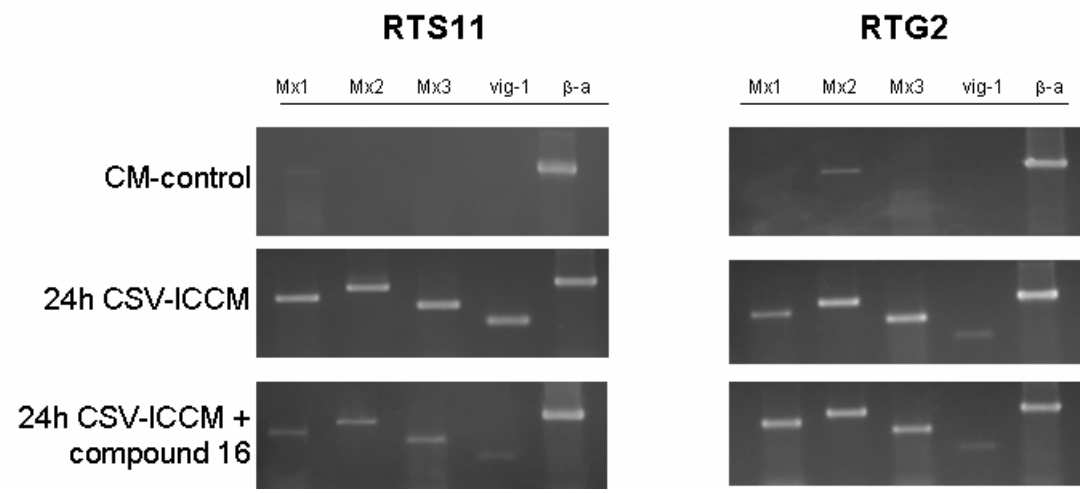

Figure 4-6. Effect of CSV-induced interferon containing conditioned media (CSV-ICCM) on *Mx* and *vig-1* expression in RTS11 and RTG-2 cultures co-treated with a PKR inhibitor.

The two cell lines were treated with CSV-ICCM with or without 0.5 μg/mL compound 16 for 24h. *Mx* and *vig-1* transcript levels were measured using RT-PCR.

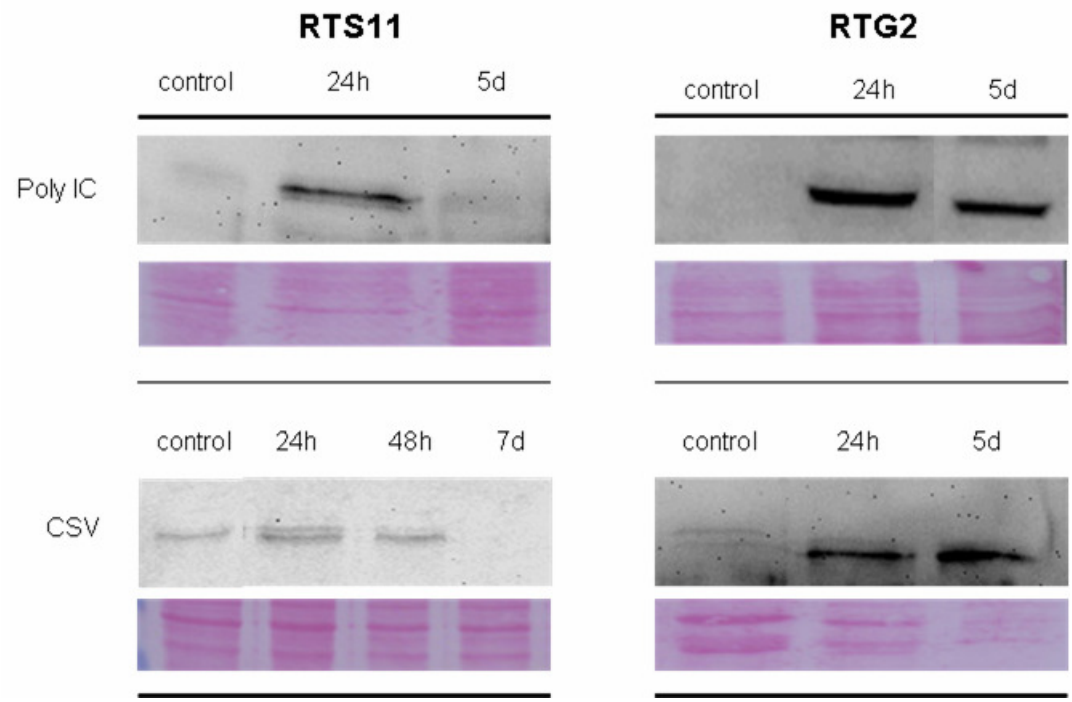

Figure 4-7. Induction of Mx expression at the protein level, following poly IC and CSV exposures.

RTS11 and RTG-2 cultures were treated with 50 µg/mL poly IC or CSV ($10^{4.3}$ $TCID_{50}$/mL) for specified lengths of time. Mx protein levels were detected using western blot analysis. Ponceau S staining of blots demonstrates equal loading of protein between samples.

Mx protein levels were also investigated in RTS11 and RTG-2 cultures after 24 h of exposure to ICCM from RTS11 and RTG-2 cultures treated with poly IC. As revealed by western blotting, Mx was not enhanced in either RTS11 or RTG-2 cultures treated with ICCM (Figure 4-8).

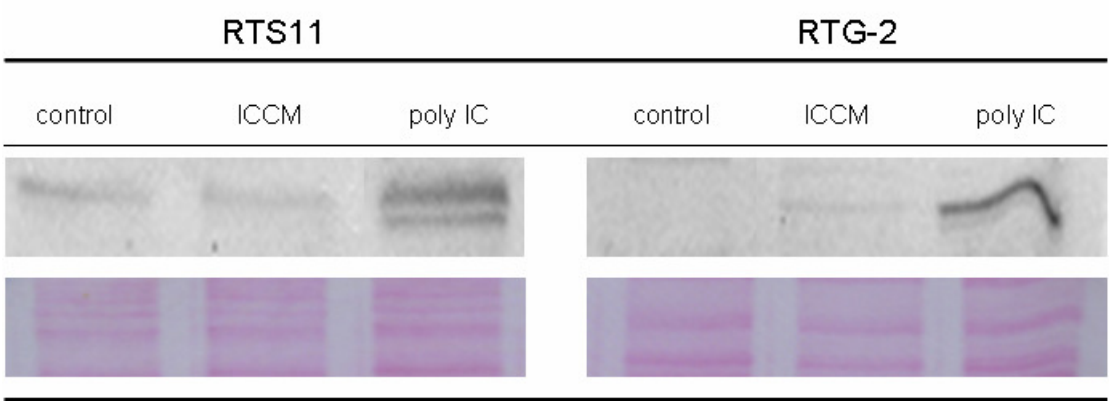

Figure 4-8. Mx protein expression levels in RTS11 and RTG-2 following exposures to ICCM.

RTS11 and RTG-2 were exposed to ICCM or 50 μg/mL poly IC for 24h. The ICCM was from RTS11 and RTG-2 cultures previously treated with poly IC. Ponceau S staining of blots demonstrates equal loading of protein between samples.

4.4. DISCUSSION

Constitutive Mx and vig-1 expression

Constitutive expression of vig-1 and Mx was low or absent in the rainbow trout fibroblast (RTG-2) and monocyte/macrophage (RTS11) cell lines. As observed previously for RTG-2 (Boudinot et al., 1999), vig-1 transcripts were not expressed constitutively, and now this has been found to be the case for RTS11 as well. Mx transcripts were rarely found to be constitutively expressed in RTG-2, but occasionally detected in RTS11. Constitutive Mx protein could be detected in RTS11 but not commonly in RTG-2 extracts, using Mx antiserum, which likely cross reacts with all three rainbow trout Mx proteins and detects a 70-72 kDa polypeptide, which is the expected size for rainbow trout Mx (Trobridge et al., 1997b). Together, this data suggests that a low level of Mx appears to be constitutively expressed in RTS11 and to a lesser extent in RTG-2. This could indicate a unique expression pattern for Mx in macrophages. Interestingly, Atlantic salmon macrophages from some fish that had not been specifically stimulated showed expression of Mx protein (Nygaard et al., 2000). *In vivo*, constitutive expression of Mx has been observed in several fish tissues (Tafalla et al., 2004; Lockhart et al., 2004; Plant and Thune, 2004; Plant and Thune, 2004)

Induction of Mx and vig-1 transcripts by poly IC and ICCM

This is the first study to demonstrate the concurrent induction of all three *Mx* genes and the *vig-1* gene by poly IC. Transcripts for *Mx1*, *Mx2*, *Mx3*, and *vig-1* were induced by 24 h in both RTG-2 and RTS11 following poly IC exposure. Previously, poly IC has been shown to induce *Mx1* transcripts by 24 h in RTG-2 (Collet and Secombes, 2001). *Vig-1* induction by dsRNA has not been previously demonstrated in any fish cell line. RTS11 might be slightly more sensitive to dsRNA, as induction of some genes could be seen as early as 4 h after poly IC exposure.

This is also the first study to demonstrate the concurrent induction of all three *Mx* genes and the *vig-1* gene by medium from cultures previously exposed to an interferon inducer, which is referred to as interferon-containing culture medium (ICCM). This induction clearly occurred in RTS11 exposed to ICCM that had been produced by poly IC stimulated RTS11. The situation with RTG-2 was more complex. When exposed to medium conditioned by control untreated cells, RTG-2 cultures expressed *Mx2* and *Mx3* transcripts, suggesting some background level of IFN production. When RTG-2 cultures were treated with medium from cells previously exposed to poly IC, no enhancement of *Mx2* and *Mx3* transcript were seen and *Mx1* and *vig-1* transcripts remained absent. The simplest interpretation for this result is that in response to a 24 h poly IC

treatment, RTG-2 cells did not produce and secrete sufficient IFN over the next 24 h to induce IFN stimulated genes (ISGs). In earlier work RTG-2 had been shown to produce ICCM after stimulation but the stimuli had been viruses or poly IC/dextran complexes (Boudinot et al., 1999; Garner et al., 2003; Collet and Secombes, 2001). These stimuli might have sustained better IFN production after their removal from RTG-2 cultures than poly IC alone.

The more rapid induction of transcripts with ICCM, as observed at 4h in RTS11, is consistent with the commonly accepted two step-process by which dsRNA induces *Mx* in fish cell cultures. The first step is the induction of interferon (IFN) synthesis and release of IFN into the medium (Collet and Secombes, 2001), which here is referred to as ICCM. When treating cells with ICCM, the IFN is present therefore only the second step would need to be executed for *Mx* induction to occur. The second step likely involves IFN triggering the Jak-STAT pathway, which consists of Janus kinases (Jak) and signal transducers and activators of transcriptions (STAT). These mediate the induction of many ISGs initially *via* ISGF3 formation (Sen, 2001) and later through IRF-1 (Platanias, 2005; Shuai and Liu, 2003). Several components of the Jak-STAT pathway have been identified in fish, including STAT1 and IRF-1 (Collet and Secombes, 2002; Zhang and Gui, 2004). IRF-1 stimulates transcription by binding an IFN-stimulating responsive element (ISRE). The promoter of rainbow trout *Mx1* contains an ISRE (Collet and Secombes, 2001), and as well, the promoter for mandarin carp *viperin* (*vig-1*) has an IRF-1 binding site (Sun and Nie, 2004).

Although both cell lines had increased dsRNA-dependent protein kinase (PKR) activity in response to poly IC as demonstrated by increased levels of phosphorylated eIF2α, PKR appeared essential for the induction by poly IC of *vig-1* transcripts in both cell lines, but only in RTS11 for *Mx*. In RTS11 and RTG-2 *vig-1* transcript induction was inhibited by two different PKR inhibitors. One inhibitor was 2-aminopurine (2-AP), which is an adenine analog, and has been the classic inhibitor of PKR (Hu and Conway, 1993), although possibly other kinases could be inhibited as well. The other, compound 16, has just recently become commercially available and is an oxindole functionalized with an imidazole and promises to be more specific as it is effective at lower concentrations (Jammi et al., 2003). For *Mx*, the two PKR inhibitors completely blocked the induction of *Mx* transcripts in RTS11 but not in RTG-2. Together the results suggest PKR has an important role, although the role of other kinases cannot be excluded. In mammalian cells an active PKR stimulates IFN synthesis through a signaling pathway that activates interferon regulatory factor-3 (IRF-3) (Williams, 2001; Peters et al., 2002; Williams, 1999).

Surprisingly, PKR appeared to have a role in the induction of *Mx* and *vig-1* in RTS11 by IFN. This is the second step in the two-step induction process. In RTS11 treated with ICCM,

PKR inhibitors blocked the induction of *Mx1* and *Mx2* completely and reduced the induction of *Mx3* and *vig-1*. In as much as the induction of *Mx* and *vig-1* by ICCM was less pronounced in RTG-2, the impact of the PKR inhibitors was less apparent. As PKR is induced in mammals by IFN (Sen, 2001), PKR levels are likely increased in RTS11 by ICCM, but as dsRNA should be absent or low, PKR must be activated by alternative mechanisms. In human and rodent cells, PKR can be activated under some circumstances in the absence dsRNA (Peel, 2004). Activated PKR might affect *Mx* and *vig-1* expression by interceding in the induction pathway by regulating the level of STAT1 and/or IRF-1. In CAB, a carp cell line, poly IC and IFN induced *STAT1* transcripts rapidly and before *Mx* transcripts (Zhang and Gui, 2004). Additionally, poly IC is known to up regulate IRF-1 in RTG-2 (Collet and Secombes, 2002). If IFN in CM induces STAT1 and/or IRF-1 in RTS11 by PKR mediated mechanisms, PKR inhibitors would keep STAT1 and/or IRF-1 levels lower and make the cells less responsive to IFN in CM.

In addition to *Mx* and *vig-1* induction by the two-step process, which can be considered an indirect mechanism, poly IC also appeared to directly induce expression of the antiviral genes in both cell lines. This is because poly IC continued to induce *Mx* and *vig-1* transcripts in the presence of cycloheximide (CHX) at a concentration that inhibited most protein synthesis in these cells (DeWitte-Orr et al., 2005b). Inhibiting protein synthesis would block the indirect process by blocking the first step, the synthesis and accumulation of IFN. A direct action by poly IC in the induction of ISG has been demonstrated in some studies with mammalian cells (Memet et al., 1991; Decker, 1992), but this is a first for a piscine system. For primate cell lines, PKR appeared to have a role in this direct action (Memet et al., 1991). In the case of RTS11 exposed to poly IC, the demonstration by 4 h of an increase in PKR activity and in some *Mx* and *vig-1* transcripts that were inhibited by compound 16 suggests that the enhanced gene expression could be due to activation by the dsRNA of a constitutively expressed PKR. This could be a third control point mediated by PKR in Mx and vig-1 induction in rainbow trout (Figure 4-9).

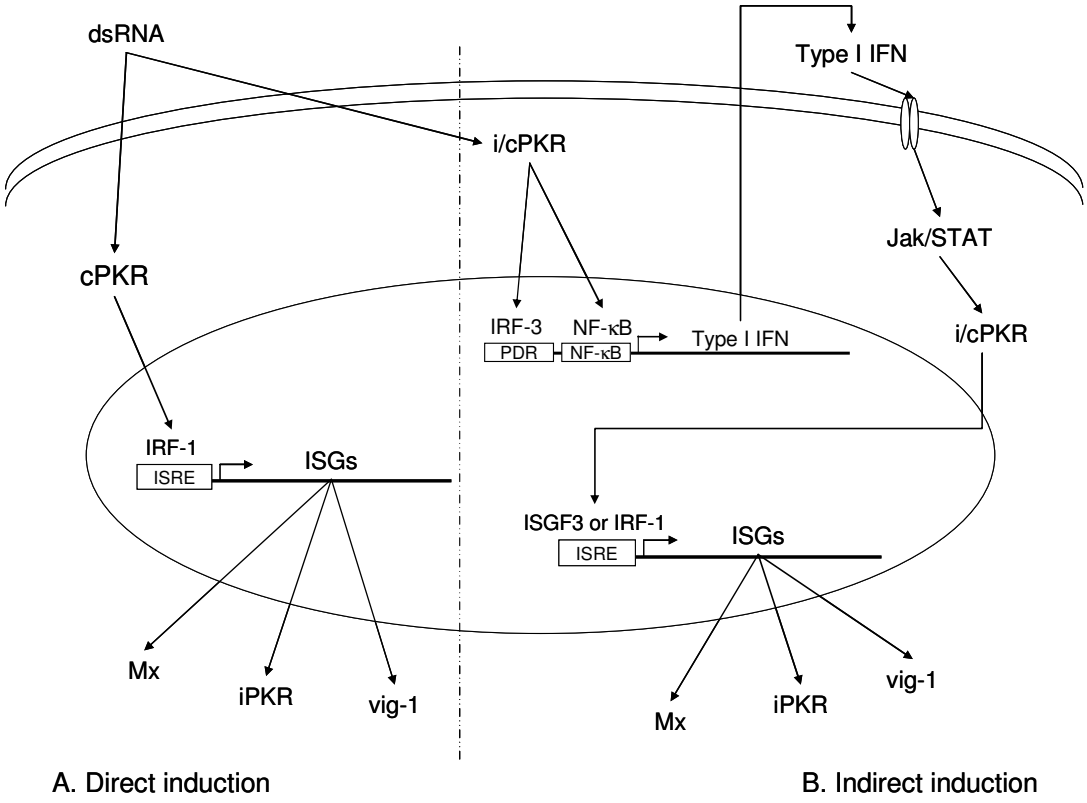

Figure 4-9. A proposed model for the pathways leading to Mx and vig-1 expression in RTS11.

Double stranded RNA (dsRNA) may be inducing ISG expression *via* direct and indirect pathways. dsRNA can be inducing ISG expression directly by activating constitutively expressed dsRNA-dependent protein kinase (cPKR), which in turn can activate IRF-1, a transcription factor that can induce ISG expression by binding to the ISRE in the promoter region of the ISGs. The indirect pathway can also involve PKR (constitutive or inducible, iPKR), inducing expression of type I IFN *via* IRF-3 and NF-κB. This IFN acts in an autocrine or paracrine fashion, binding to its cognate receptor and signaling through the Jak/STAT pathway to induce ISG expression through ISGF3 formation or IRF-1 activity also *via* PKR. Abbrevations: **IFN** – interferon, **ISG** - interferon stimulated gene, **IRF** - interferon regulatory factor, **ISRE** - interferon stimulated response element.

Induction of Mx and vig-1 transcripts by CSV

This is the first demonstration in fish cell lines of *Mx* and *vig-1* induction by an aquareovirus (AqRV) and of concurrently induced expression of the three *Mx* genes and a *vig-1* gene by a virus. CSV belongs to subgroup A among the AqRV, and the only other AqRV to be studied for the capacity to induce *Mx* has been channel catfish virus (CCV), which belongs to the AqRV-D (Essbauer and Ahne, 2001). Injection of CSV induced an *Mx* transcript in the liver of channel catfish (Plant and Thune, 2004). Most studies on Mx induction by viruses *in vitro* have involved rhabdovirues, which unlike AqRV have a membrane. Several rhabdoviruses have been examined, including viral haemorrhagic septicemia virus (VHSV), Hirame rhabdovirus (HRV),

and grass carp haemorrhagic virus (GCHV) (Boudinot et al., 1999; Zhang et al., 2004; Lin et al., 2005). In RTG-2, VHSV was shown to induce a transcript for *Mx* but not for *vig-1* (Boudinot et al., 1999). GCHV induced transcripts for two *Mx* genes in the carp cell line, CAB, but CHX blocked induction of one gene and not the other, implying different mechanisms of regulation (Zhang et al., 2004). By contrast, in the rainbow trout cell lines, the three *Mx* genes responded the same way, although differently in RTS11 than in RTG-2.

In RTS11, CSV appeared to induce *Mx* and *vig-1* transcripts indirectly *via* the induction of IFN, which could accumulate in the medium to form ICCM, and directly by a process independent of ICCM. Supporting the indirect action is the observation that ICCM prepared from RTS11 cultures previously exposed to CSV was able to induce *Mx* and *vig-1* in RTS11. Supporting the direct action is the observation that *Mx,* but less so with *vig-1,* transcripts were induced in the presence of CHX, which would have prevented the formation of ICCM and an indirect action. Both mechanisms of induction appeared to require viral RNA synthesis as induction of *Mx* and *vig-1* transcripts was blocked by ribavirin. For mammalian cells, ribavirin has been shown to inhibit the transcription of viral genes for reoviruses with little effect on host cell RNA synthesis (Rankin et al., 1989). Ribavirin is presumed to act similarly with CSV and the rainbow trout cell lines, and indeed, ribavirin did not prevent *Mx* and *vig-1* induction in RTS11 by poly IC (data not shown). As PKR inhibitors blocked induction, PKR and viral dsRNA appear to mediate the induction of *Mx* and *vig-1* transcripts in RTS11.

In RTG-2, CSV appeared to act primarily *via* an indirect mechanism, requiring the induction of IFN first to induce *Mx* and *vig-1* transcripts. This is supported by the observation that induction was almost completely blocked by CHX, which would block IFN synthesis. As well, the medium conditioned by RTG-2 previously exposed to CSV, and presumed to contain IFN, was able to induce *Mx* and *vig-1* in RTG-2. In contrast to RTS11, the induction of *Mx* and the presumed inducer, IFN, did not require CSV RNA synthesis as *Mx* but not *vig-1* induction continued in RTG-2 cultures with ribavirin. Also in contrast to RTS11, the induction of *Mx* transcripts in RTG-2 appeared to be independent of PKR as induction occurred in the presence of PKR inhibitors. In the mammalian literature, some examples exist of viruses or viral components inducing IFN independent of PKR (Stewart et al., 2003). Possibly a CSV protein(s) rather than RNA induces IFN and in turn *Mx* in RTG-2.

For reovirus infection of mammalian cells, the conversion of the capsid to intermediate subviral particles (ISVP) is an essential step and the lysosomal proteases responsible for this can differ between macrophages and fibroblasts (Golden et al., 2004). As well as entering by endocytosis, ISVP can under some circumstances penetrate cell membranes (Borsa et al., 1979;

Tosteson et al., 1993). Little is known about these steps in aquareoviruses, but possibly, CSV virions are processed and/or enter RTS11 and RTG-2 differently, leading to different pathways for *Mx* and *vig-1* induction.

Induction of Mx protein

Treatment with poly IC or CSV caused Mx protein accumulation in RTS11 and RTG-2 cultures, but differences were found between the cell lines. Previously, a 48 h poly IC treatment had been shown to induce Mx in RTG-2 (Trobridge et al., 1997a), and when the inducer was a complex of poly IC and dextran, Mx levels had begun to decline by 72 h (Trobridge et al., 1997a). In the current study, the induction of Mx protein by poly IC was seen by 24 h with both RTS11 and RTG-2, and longer treatments showed a clear difference between the cell lines. In RTS11 the Mx level declined to near control levels by 5 days, while Mx protein induction was still evident in RTG-2. For Mx protein induction by viruses in salmonid cell lines, ISAV but not IPNV induced Mx in SHK-1 and TO (Jensen and Robertsen, 2002) and IHNV failed to induce in RTG-2 (Trobridge et al., 1997a). In this study, CSV induced Mx protein by 24 h in both RTS11 and RTG-2. However, the Mx level declined thereafter in RTS11 but remained high in RTG-2 cultures. It is possible that posttranslational regulation of Mx differs in RTS11 and RTG-2.

The lack of induction by ICCM of Mx protein in RTS11 cultures despite the induction of *Mx* transcripts could have several causes. One possibility is that the kinetics of Mx protein accumulation and the decline are different with ICCM than with poly IC and the induction was missed. Another possibility is that the amount of IFN in ICCM was inadequate to sustain Mx protein induction long enough to see accumulation detectable by western blot analysis.

In this study expression of antiviral proteins, Mx and vig-1, were compared at the transcript and protein level following treatment with dsRNA and live virus between the macrophage-like RTS11 and fibroblast RTG-2. Clearly expression patterns and induction pathways varied between the two cell lines. These differences may reflect differences in cell type between the two cell lines, perhaps suggesting differences in the regulation of antiviral mechanisms between immune and non-immune cells. Alternatively, non-immune functions for these proteins, such as affecting cell metabolism or intracellular protein localization, may also contribute to differences between the two cell lines.

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Chapter 5

Induction of Homotypic Aggregation in the Rainbow Trout Macrophage-like cell line, RTS11

ABSTRACT

Understanding how to manipulate the fish immune system is important for the development of aquaculture. One immune response that potentially could be modulated but is poorly understood in fish is homotypic aggregation (HA). HA is a characteristic of activated leukocytes and plays an important role in the inflammatory response, particularly for the migration of neutrophils and macrophages to the site of infection. In this study homotypic aggregation was induced in RTS11, a rainbow trout macrophage-like cell line, by a number of immunological stimuli including: poly inosinic:cytidylic acid (poly IC), poly adenylic acid (poly A), lipopolysaccharide (LPS), zymosan, and phorbol 12-myristate 13-acetate (PMA). These stimuli mimic viral, bacterial and fungal infections or can directly activate macrophages respectively, thus suggesting that HA in RTS11 may indicate a general response mechanism of an activated macrophage. Poly IC, which has been previously shown to activate antiviral mechanisms in this cell line, was found to induce the strongest HA response. Poly IC-induced homotypic aggregation in RTS11 was found to be dose- and time-dependent. This process was found to require active cell metabolism as well as calcium and magnesium, indicative of integrin or perhaps galectin involvement. Interestingly, PKR, a dsRNA-dependent protein kinase, was found to be involved in this pathway as two inhibitors completely abrogated poly IC- induced aggregation. PKR is a pivotal antiviral protein in mammals and its involvement in this pathway suggests that homotypic aggregation is an important antiviral response in fish.

5.1. INTRODUCTION

Homotypic aggregation (HA), the active adherence of like cell types to one another, is an important immune cell response to pathogenic stimuli. HA has been observed in many immune cell types, including: monocytes (Yue et al., 1999), neutrophils (Rochon and Frojmovic, 1992), lymphocytes (Zapata et al., 1995) and eosinophils (Teixeira et al., 1995). It has been suggested that this cellular adhesion is important for leukocyte trafficking (Cho et al., 2003) and is a characteristic of activated immune cells (Teixeira et al., 1995). In fact, HA has been used as a marker of activated immune cells *in vitro*, and has been shown to affect cytokine expression and cellular proliferation (Wake et al., 1995).

Monocyte/macrophages play an important role in the innate immune response against invading pathogens. Circulating monocytes become activated at a site of inflammation and enter the infected tissue, differentiating into macrophages. HA is often observed following monocyte activation (Frostegard et al., 1990; Hmama et al., 1999). It is this cell-cell adhesion that may aid in the migration and differentiation of monocytes to sites of infection (Watanabe and Fan, 1998). A similar phenomenon is seen with neutrophils, where cells adhered to the vessel wall may aid in the recruitment of circulating neutrophils, this HA contributes to cell arrest in the blood vessel (Neelamegham et al., 2000; Kunkel et al., 1998). Human monocytes are able to undergo HA *in vitro* in response to PMA (Yue et al., 1999), interferon-γ treatment (Mentzer et al., 1986), oxidized low density lipoprotein (Frostegard et al., 1990) and staurosporine (Cho et al., 2003). Macrophage-like cells have also been shown to undergo HA *in vitro* (Moese et al., 2002).

Viruses can also induce HA *in vitro* and it has been suggested that HA may play an important role in persistence of viral infection (Takahashi et al., 2002). For example, latent Epstein-Barr virus infections in B lymphocytes trigger HA (Park and Faller, 2002) and the human T-cell leukemia virus type I (HTLV-1) Tax protein induces HA in human T cells (Takahashi et al., 2002). Poly IC, a synthetic dsRNA and viral mimic, has also been shown to upregulate adhesion molecule expression and induce leukocyte adhesion in endothelial cells independent of type I interferon (Faruqi et al., 1997).

In mammals, B cell (Zapata et al., 1995), T cell (Wake et al., 1995), monocyte (Kasinrerk et al., 1999) and macrophage (Moese et al., 2002) HA is mediated through the integrin leukocyte function-associated antigen (LFA)-1 interacting with its counter-receptor, the Ig superfamily member intercellular adhesion molecule (ICAM)-1. Poly IC can induce ICAM-1, VCAM-1 (Yang et al., 1994; Marui et al., 1993) and E-selectin (Bandyopadhyayk et al., 2000) expression in endothelial cells and ICAM-1 expression in dendritic cells (Smits et al., 2002). It has also been

shown that PKR, the dsRNA-dependent protein kinase, can mediate expression of poly IC-induced adhesion molecules (Offermann et al., 1995).

Very little is known of adhesion molecules in fish; however, there are indications of integrin involvement in fish cell adhesion. Evidence for an integrin fibrinogen receptor has been reported in thrombocytes (Hill and Rowley, 1998) and fibroblasts (Reinhart and Lee, 2002). Viral hemorrhagic septicemia virus (VHSV) G-protein induced Ig superfamily member, B lymphocyte cell adhesion molecule (BLCAM), expression in Japanese flounder juveniles (Byon et al., 2005). HA has been reported in channel catfish neutrophils stimulated with phorbol dibutyrate (PBDU) (Ainsworth et al., 1996). Neutrophil adhesion was suggested to be mediated by integrins as it was divalent cation-dependent and could be blocked with the addition of the peptide RGD (Arg-Gly-Asp), an integrin binding sequence.

In this study, HA was investigated in RTS11, a rainbow trout monocyte/macrophage cell line. A strong HA response could be induced in RTS11 following exposures to poly IC but only slightly with a synthetic ssRNA, poly A, suggesting HA is an antiviral response in RTS11. This is the first report of poly IC induced HA *in vitro*. Slight HA could be detected following treatments with the classic mammalian HA inducer, phorbol 12-myristate 13-acetate (PMA), gram negative bacteria cell wall component, lipopolysaccharide (LPS), and a yeast cell wall component, zymosan, suggesting that HA could also be a general marker for activated macrophages *in vitro*. Poly IC-induced HA was further investigated and was found to be a cell response requiring active cell metabolism and an intact cytoskeleton, indicating aggregation instead of passive cell agglutination. Poly IC-induced HA was divalent cation dependent, suggesting the role of integrins or perhaps galectins in cell aggregation. PKR, the dsRNA-dependent protein kinase, was found to play in important role in poly IC-induced HA in RTS11.

5.2. MATERIALS AND METHODS

5.2.1. Cell culture

RTS11, a rainbow trout monocyte/macrophage cell line (Ganassin and Bols, 1998), was grown in L-15 medium (Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS), 150U /mL penicillin G, and 150µg/mL streptomycin sulfate (Sigma). RTS11 cells were grown at 18°C in 25cm^2 tissue culture treated flasks (Nunc, Roskilde, Denmark) and passaged every three to four weeks, the culture and conditioned medium was split into two flasks, and an equal volume of fresh medium was added to each flask.

5.2.2. Cell treatments

RTS11 were seeded into 96 well plates (Falcon/Becton-Dickinson, Franklin Lakes, NJ) in full growth medium at 1.5x10^5 cells per well and allowed to attach overnight at 18°C. Cells were then treated with the stimuli. Unless specified, cell aggregates and cell viability were generally measured after 24 hours of incubation at 18°C.

Chemical Stimuli

Poly inosinic: poly cytidylic acid (poly IC; 0.05-500 µg/mL), poly adenylic acid (poly A; 0.05-500 µg/mL), lipopolysaccharide (LPS; 0.1-100 µg/mL), zymosan (0.005-50 µg/mL), and phorbol 12-myristate 13-acetate (PMA; 0.2 ng-2 µg/mL) were added to the cells in increasing concentrations, with 6 replicates per concentration. All chemical stimuli were purchased from Sigma, with the exception of zymosan, which was purchased from InvivoGen (San Diego, CA). Cells were also treated with the carrier alone, in cases when the solvent was not L-15. In no cases was the solvent used at a concentration that was cytotoxic. Generally, cells were treated with the compounds for 24h before cell viability and HA were measured.

Inhibitors

All chemical inhibitors were added just prior to addition of the stimulant. The inhibitors used in this study include: cycloheximide (CHX; 0.1 & 1 µg/mL), actinomycin D (AMD; 0.05 & 0.5 µg/mL), colchicine (Col; 5-500 µg/mL), latrunculin B (Lat; 0.05 & 0.5 µg/mL). Two PKR inhibitors were used in this study, 2-aminopurine (2-AP; 1.5mM) and a commercially available oxindole functionalized with an imidazole, referred to as compound 16 (Jammi et al., 2003)(0.5 µg/mL) with its negative control (0.5 µg/mL). Two integrin inhibitors were also used, RGD (5µg/mL) and an LFA-1 inhibitor (2.5-25 µg/mL). All of the inhibitors were purchased from

Sigma, with the exception of compound 16, its negative control, RGD and LFA-1 inhibitor which were purchased from Calbiochem (La Jolla, CA). As mentioned above, control cells were also treated with the carrier alone, in cases when the solvent was not L-15. In no cases was the solvent used at a concentration that was cytotoxic. When low temperature was used as an inhibitor, the cultures were dosed with poly IC and immediately placed at 4°C. The cultures were kept at this temperature for 24h.

Ca^{2+}/Mg^{2+}- free conditions

When measuring divalent cation dependence, the cells were washed twice in Ca^{2+}/Mg^{2+}- free Hank's buffered saline solution (HBSS; Sigma) before being plated at $1.5x10^5$ cells per well in Ca^{2+}/Mg^{2+}- free HBSS. No FBS was added to these cultures to ensure Ca^{2+}/Mg^{2+}- free conditions. RTS11 was also seeded using HBSS with Ca^{2+} and Mg^{2+} as a control. Divalent cation dependence was also evaluated by treating RTS11 with poly IC in combination with 5mM EDTA in serum free L-15 growth medium. No cell death was detected under these conditions as measured using the two fluorescent indicator dyes, alamar Blue and CFDA-AM, as described below (data not shown).

5.2.3. Quantifying homotypic aggregation (HA)

RTS11 cells in 96-well plates were examined for homotypic cell aggregation under a phase-contrast microscope. Representative pictures of each well were taken at a magnification of 200X, therefore 6 pictures were taken per treatment. Using Adobe Photoshop, aggregates larger than a 0.04 mm by 0.04 mm square were counted. In general, this particular size marker would include between 15 to 25 cells per aggregate. Any aggregate that did not fill the square was not included in the count. A summary of other published HA quantification methods are listed in Appendix A.

5.2.4. Measuring cytotoxicity

Two fluorescent indicator dyes, alamar Blue™ (Medicorp, Montreal, PQ) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Molecular Probes, Eugene, OR) were used to evaluate cell viability. Viable cells cause the reduction of alamar Blue dye resulting in a chemical change from a non-fluorescent, blue form (resazurin) to a fluorescent red form (resorufin) (O'Brien et al., 2000). CFDA-AM, which is also taken up by live cells, is hydrolyzed by non-specific intracellular esterases to yield the fluorescent form, carboxyfluorescein. These esterases are only active within the confines of a cell; therefore, CFDA-AM can be used to monitor cell membrane integrity (Schirmer et al., 1997). The levels of fluorescence are measured using a microplate spectrofluorometer. Alamar Blue and CFDA-AM assays were performed as

previously described (Ganassin et al., 2000). Briefly, the cells were treated as described above. After incubation for the described amount of time, the media was removed and the two fluorescent dyes were added to the wells, diluted in a minimal salt solution called L-15ex. The cells were incubated with the dyes for 60 minutes before the microwells were read with a fluorometric plate reader (Molecular Devices SpectraMax). Excitation and emission wavelengths were 530 and 595 nm for alamar Blue and 485 and 530 nm for CFDA-AM, respectively.

5.2.5. Data analysis

For alamar Blue and CFDA-AM assays, cytotoxicity was indicated by a decline in fluorescence units (FUs) for experimental cultures relative to control cultures. FUs for culture wells with no cells were constant and subtracted from FUs for experimental and control cultures. For graphical presentation, the results were plotted using SigmaPlot (Jandel Scientific). When comparing the effect of the inhibitors on aggregate formation, the average number of aggregates per treatment was compared to the control cultures with poly IC alone using an unpaired t-test. All statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com).

5.3. RESULTS

5.3.1. Homotypic aggregation (HA) as a general anti-pathogenic response in RTS11

After 24h exposures, poly IC, a synthetic dsRNA and viral mimic, induced a dramatic change in culture morphology known as homotypic aggregation (Figure 5-1). A synthetic ssRNA, poly A, induced a small amount of aggregation at high concentrations, suggesting specificity to dsRNA.

HA in RTS11 was not limited to viral stimuli. Phorbol 12-myristate 13-acetate (PMA), a PKC activator and classic HA inducer in mammalian cells, also induced HA in RTS11. Lipopolysaccharide (LPS), a cell wall component of the gram-negative bacterium *Escherichia coli*, and zymosan, a cell wall component of the yeast *Saccharomyces cerevisiae* both induced HA in RTS11 as well (Figure 5-1).

It is important to note that although various stimuli induced HA in RTS11, the morphology of the aggregates differed. Poly IC-, LPS- and zymosan-stimulated aggregates were 'stellate' in morphology, while Poly A-, and PMA- stimulated aggregates were a more 'globular' morphology.

HA induced by these stimuli was quantified, and poly IC was found to be the most dramatic inducer of HA in RTS11 after 24h exposures (Figure 5-2), while poly A induced a small amount of HA at high concentrations. HA induction was also observed following treatments with PMA, zymosan and LPS, with varying responses between stimuli. In all treatments, aggregation became stronger over days of exposure, with the exception of poly IC, which stimulated a maximum number of aggregates by 24h. After 24h, poly IC-induced aggregates became larger and more pronounced but no new aggregates formed (data not shown).

Arguably, the change in culture morphology following stimuli treatment could be interpreted as the result of cell death; however, none of the stimuli tested killed RTS11 (Figure 5-3). Thus, HA induced by all stimuli tested was not a result of cell death but of cell migration and aggregation. Cell viability was measured using two fluorescent indicator dyes, alamar Blue and CFDA-AM, which measure cell metabolism and membrane integrity respectively.

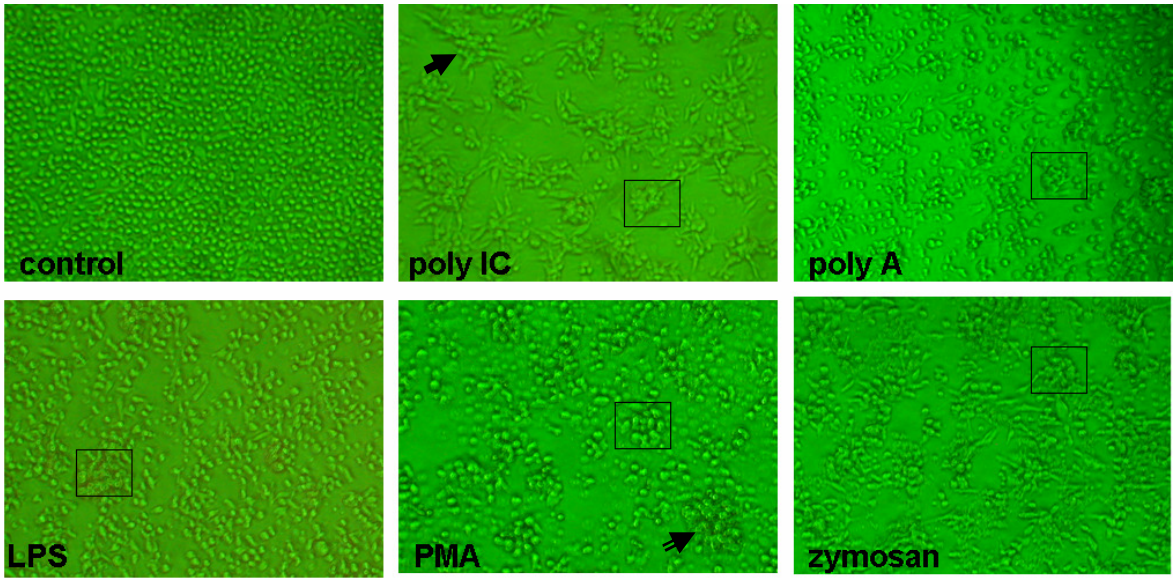

Figure 5-1. Induction of homotypic aggregation (HA) in RTS11 cultures.

After 24 h, aggregates were observed in RTS11 cultures treated with 5 µg/mL poly IC, 5 µg/mL poly A, 100 µg/mL LPS, 2 µg/mL PMA, or 50 µg/mL zymosan for 24h in full growth media. A box in each picture highlights an aggregate. Aggregate morphology varied depending on the stimulus, for example, poly IC induced 'stellate' –like aggregates (indicated by a single arrow), while PMA induced 'globular'-like aggregate morphology (double arrow). After 24h, poly IC treated cultures showed the most dramatic difference culture morphology; the number of aggregates in each treatment is quantified in Figure 5-2. Pictures were taken at 200X magnification.

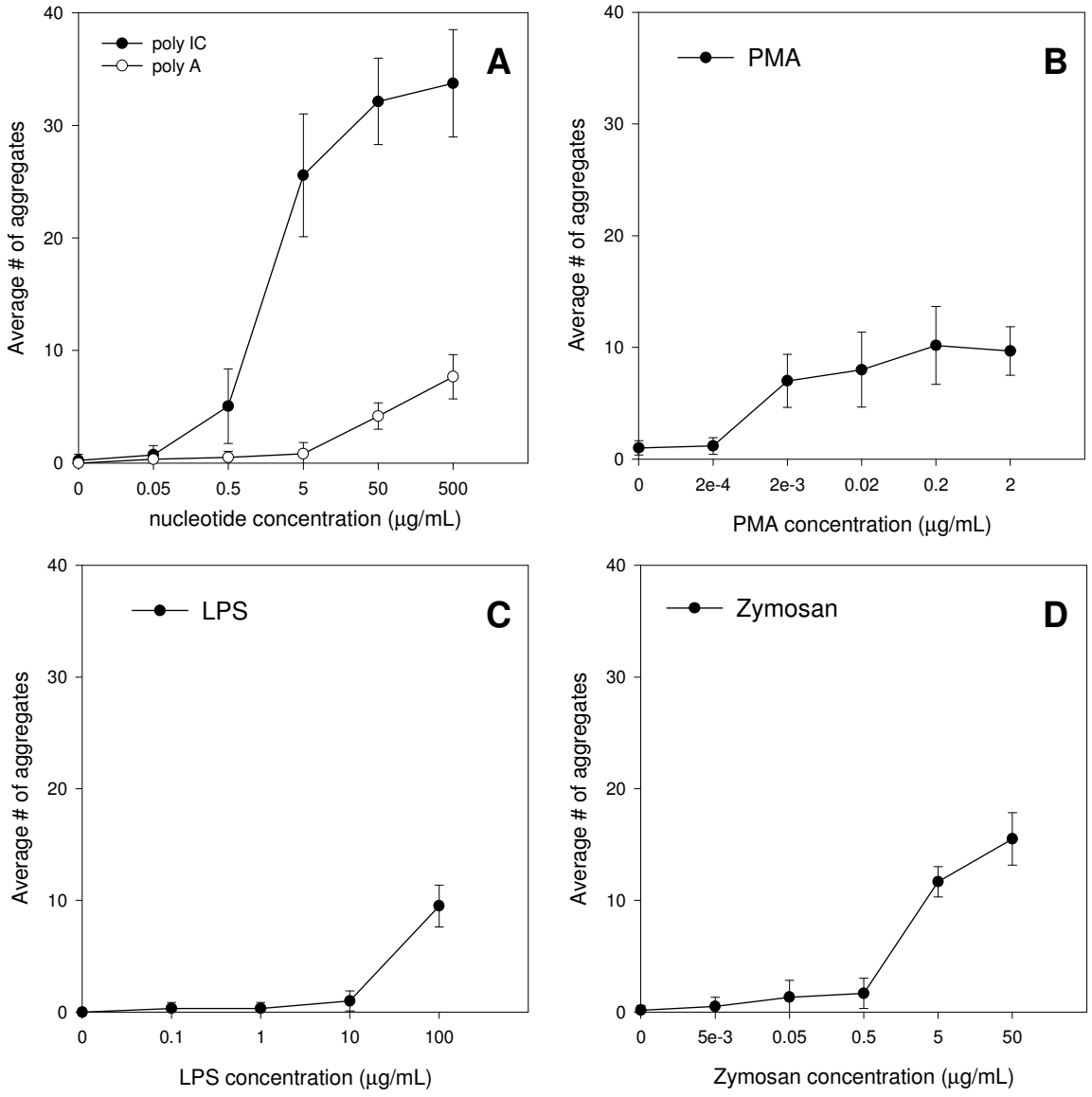

Figure 5-2. Quantification of HA induction by different inducers.

RTS11 cultures were exposed for 24h to synthetic dsRNA, poly IC (**A**), a synthetic ssRNA, poly A (**A**), PMA (**B**), LPS (**C**), and zymosan (**D**) and evaluated for HA as described in Materials and Methods.

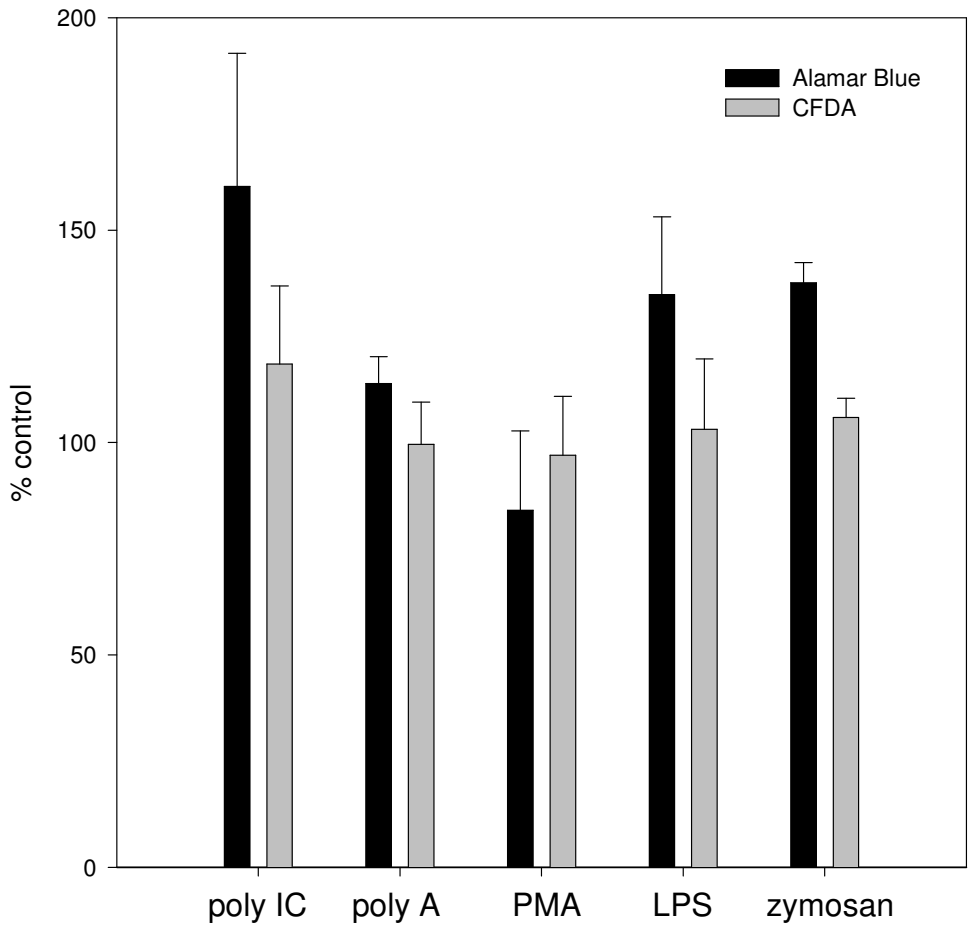

Figure 5-3. Effect of treatments causing HA on RTS11 cell viability.

RTS11 cultures were treated with 5 µg/mL poly IC, 5 µg/mL poly A, 2 µg/mL PMA, 100 µg/mL LPS, or 50 µg/mL zymosan, and after 24 h cell viability was measured with alamar blue and CFDA-AM. All values are expressed as % of carrier treated controls, and are plotted on the y-axis.

5.3.2. Poly IC induced HA is time and concentration dependent

As poly IC proved to be the most powerful HA inducer after 24h and is an effective antiviral stimulant in RTS11 (DeWitte-Orr et al., 2005), it was selected as a model stimulant to further elucidate HA mechanisms in RTS11. Poly IC-induced HA was concentration dependent, as more aggregates could be observed in cultures treated with higher concentrations of poly IC. HA was also determined to be dependent on time, as the number of aggregates increased over time (Figure 5-4). As mentioned above, after 24h the number of aggregates did not increase; however, the size of each individual aggregate continued to increase over time (data not shown).

5.3.3. HA in RTS11 is an active cell response

Poly IC induced aggregation in RTS11 was determined to be an active cell response, in contrast to the passive response of agglutination. Metabolic inhibitors, such as low temperature and inhibitors of translation (cycloheximide) and transcription (actinomycin D) were found to block poly IC- induced HA in a concentration dependent manner (Figure 5-5). A previous study found that in RTS11, 0.1 μg/mL cycloheximide and 0.5 μg/mL actinomycin D were responsible for inhibiting the majority of ^{3}H-leucine and ^{3}H-urindine incorporation respectively (DeWitte-Orr et al., 2005). Thus *de novo* protein synthesis is required for an effective HA response in RTS11.

Cytoskeleton inhibitors, colchicine and latrunculin B also blocked HA in RTS11 in a concentration dependent manner (Figure 5-5). Colchicine (Col) and latrunculin B (Lat) disrupt microtubule and microfilament-mediated process respectively, suggesting that cytoskeleton integrity or reorganization is essential for HA in RTS11.

5.3.4. Ca^{2+}/Mg^{2+} dependence in poly IC induced HA

Commonly, the role of integrins in HA has been demonstrated by the dependence of aggregate formation on the presence of divalent cations. It was found that cells treated with poly IC in media without Ca^{2+} and Mg^{2+} were unable to aggregate (Figure 5-6). Treatment with poly IC and 5mM EDTA in normal growth media also inhibited, but did not completely abrogate, aggregation (data not shown). Involvement of specific integrins was investigated; however, poly IC-induced HA in RTS11 was not inhibited using RGD or an LFA-1 specific inhibitor (data not shown).

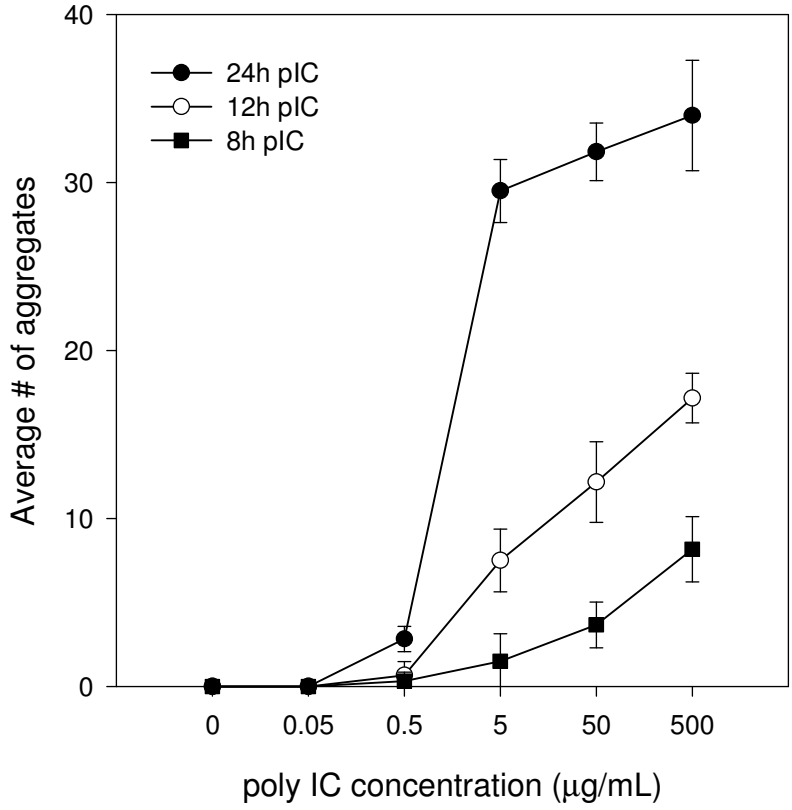

Figure 5-4. Effect of time and poly IC concentration on HA.

RTS11 cells were treated with increasing concentrations of poly IC and monitored over time for homotypic aggregation.

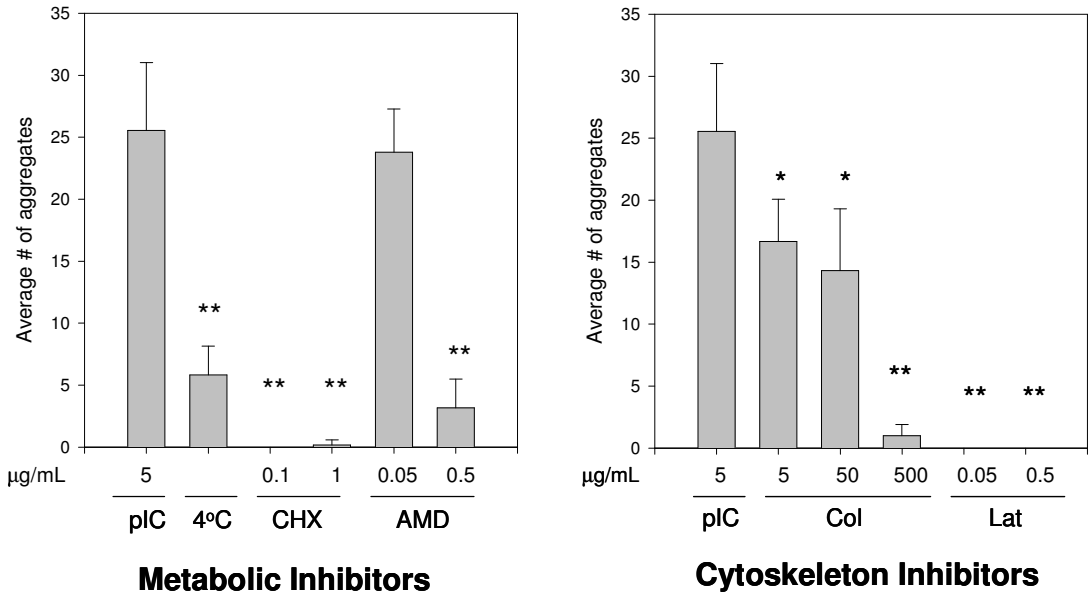

Figure 5-5. Effect of metabolic and cytoskeleton inhibitors on poly IC-induced HA.

RTS11 were exposed to 5µg/mL poly IC in combination with inhibitors for 24h in full growth media. The metabolic inhibitors included low temperatures, where cells were treated with poly IC and immediately placed at 4°C for 24h. RTS11 were also treated with translation and transcription inhibitors, cycloheximide (CHX) and actinomycin D (AMD) respectively. Two cytoskeleton inhibitors, colchicine (Col) and latrunculin B (Lat) which disrupt microtubule and microfilament-mediated processes, were also used in combination with poly IC treatment. These results represent three similar experiments. Treatments were compared using an unpaired t-test, all values which differed significantly from controls are indicated by two asterisks ($P < 0.0001$) or by one asterisk ($P < 0.001$).

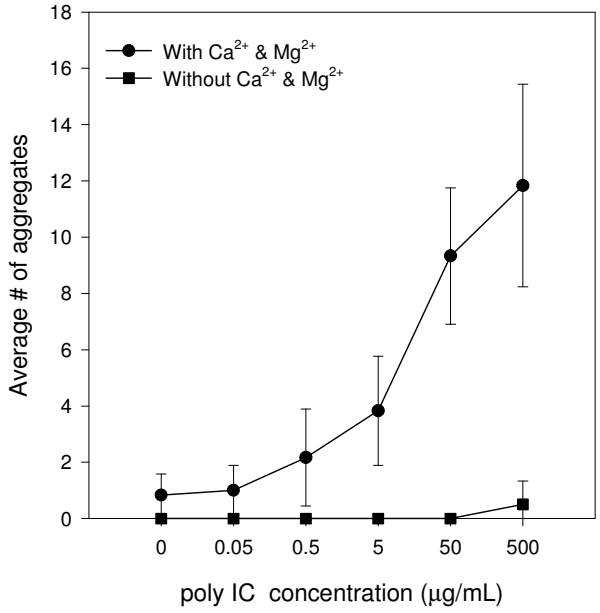

Figure 5-6. Effect of calcium and magnesium on poly IC-induced HA.

RTS11 were seeded in Hanks' balanced salt solution (HBSS) with or without 0.185 g/L $CaCl{\cdot}2H_2O$ and 0.098 g/L $MgSO_4$(anhyd) and dosed with increasing concentrations of poly IC.

5.3.5. Poly IC induced HA involves PKR

Poly IC signaling pathways have previously been shown to involve PKR in RTS11 (DeWitte-Orr et al., 2005), thus the involvement of this important antiviral kinase in poly IC-stimulated HA was investigated. PKR involvement was demonstrated when two inhibitors, 2-aminopurine and compound 16, both blocked poly IC-induced HA (Figure 5-7).

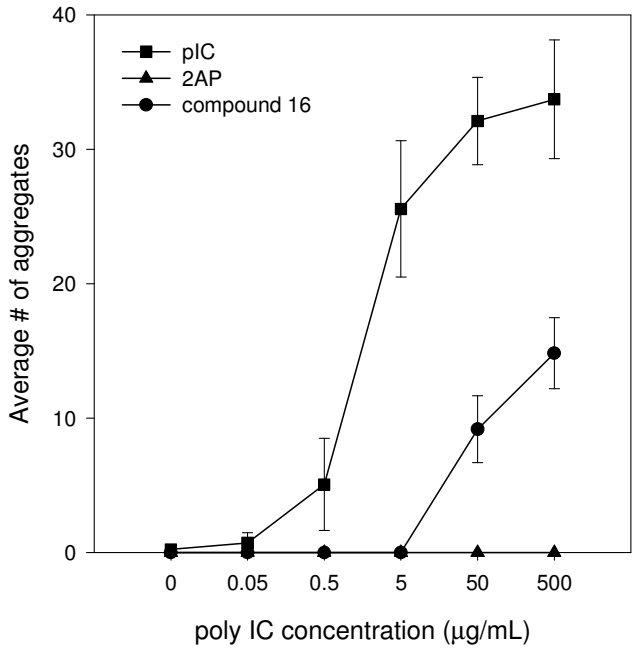

Figure 5-7. Effect of PKR inhibitors on poly IC- induced HA.

Two PKR inhibitors, 2-aminopurine (2-AP) and compound 16, blocked poly-IC induced aggregation in RTS11 after 24h exposure. The negative control for compound 16 did not affect poly IC-induced HA (data not shown).

5.4. DISCUSSION

HA as an indicator of activated macrophages

Poly IC, a viral mimic, was able to induce HA in RTS11. A synthetic ssRNA, poly A, was also able to induce HA but not to the same extent of dsRNA. These results suggest that HA may be part of the macrophage antiviral response mechanism. Previous studies have shown that poly IC induces other antiviral mechanisms in RTS11, such as apoptosis and antiviral gene expression (DeWitte-Orr et al., 2005) (chapter 4). Poly IC-induced HA was concentration dependent and required 24h for maximal aggregate formation, suggesting that pathways were being activated over time that ultimately resulted in HA.

HA was not limited to viral products and was induced in RTS11 following treatments with PMA, LPS, and zymosan. In no case did any of the stimuli tested cause cell death. Thus, HA seems to be a general response to immunological stimuli and may represent a good marker for monocyte/macrophage activation *in vitro*. Previous studies have suggested that HA is strongly associated with monocyte activation (Moese et al., 2002). Frostegard *et al.* demonstrated that with both the human monocytic cell line, U937, and primary human monocytes, HA correlated directly with monocyte activation following treatment with PMA and oxidized low density lipoprotein (Frostegard et al., 1990). Thus, HA may serve as an easily identifiable indicator of activation, perhaps for screening purposes.

It is interesting to note that although HA may be a general response of macrophages to immunological stimuli, aggregate morphology differed between stimuli. Poly IC-, LPS- and zymosan-stimulated aggregates were 'stellate' in morphology, while Poly A- and PMA-stimulated aggregates had a 'globular' morphology. Both shapes can be found in the literature, with stellate aggregates being present in the stimulated human macrophage cell line, K1m (Dialynas et al., 1997) and globular morphology being common in PMA-stimulated U937 cells (Yue et al., 1999). It does not appear that these differences in aggregate shape have ever been described within the same cell line. It is possible that different stimuli trigger expression of different adhesion molecules, resulting in distinct aggregate morphology. It is interesting to note that PMA induced a globular aggregate morphology in RTS11 as well as in human monocytic cells (Yue et al., 1999; Hong et al., 2003).

As poly IC was the strongest inducer of HA tested, it was therefore used as a model stimulant to further elucidate HA in RTS11.

HA is an active response

Cell clumping can occur by two processes: agglutination and aggregation. Cell agglutination is a passive process whereby membrane proteins or glycoproteins adhere to each other, resulting in cell clumping. Aggregation is an active process that requires cell activation, shape change, and adhesion (Chow and Kini, 2001). Poly IC-induced cell clumping was determined to be aggregation as defined by several different parameters.

Firstly, poly IC-induced HA required an active cell metabolism, as translation and transcription inhibitors, cycloheximide (CHX) and actinomycin D (AMD), blocked HA in a concentration dependent manner. Incubating the cells at 4^{o}C while treating with poly IC also inhibited HA, showing that slowing down metabolism by lowering the incubation temperature would also block HA. The fact that poly IC-induced HA does not appear in RTS11 cultures until at least 8h of exposure provides further support that protein synthesis and an active cell response was required for HA.

Secondly, poly IC induced HA was also found to be dependent on an intact and functioning cytoskeleton, as colchicine and latrunculin B, which disrupt microtubules and microfilament-mediated processes respectively, also inhibited HA in a dose dependent manner. These results agree with HA in gilthead seabream macrophages, which were also found to be dependent on active cell metabolism and an intact cytoskeleton (Mulero et al., 2001).

Thirdly, poly IC-induced HA was dependent on the presence of divalent cations. Poly IC did not induce HA in RTS11 when grown in medium without Ca^{2+} and Mg^{2+}, aggregation was also partially blocked in full media with the addition of EDTA. Channel catfish neutrophil and gilthead seabream macrophage HA were also found to be divalent cation dependent (Ainsworth et al., 1996; Mulero et al., 2001). This dependence suggests the involvement of a small number of adhesion molecule families that require divalent cations to function. Taken together, these results suggest that poly IC is acting by increasing expression and activation of adhesion molecules, causing a change in cell morphology, resulting in aggregation.

Adhesion molecule involvement

As mentioned above, poly IC-induced HA in RTS11 was found to be dependent on the presence of divalent cations. Based on this data, integrins were suspected to be the adhesion molecules involved. In mammals, monocyte HA is most commonly reported to be mediated through the integrin LFA-1 and the Ig superfamily member, ICAM-1 (Weeks and Iuorno, 1996; Mentzer et al., 1986), whose interactions are known to be divalent cation dependent (Rothlein and Springer, 1986). Also, poly IC has been shown to upregulate ICAM-1 expression in dendritic

cells (Smits et al., 2002) and the measles virus can alter LFA-1, triggering leukocyte aggregation (Attibele et al., 1993). Two inhibitors were used to identify the adhesion molecules involved in poly IC induced HA.

RGD is a tri-peptide recognition sequence which binds to specific integrins and can be used to block fibrinogen binding to human monocytes. RGD has been shown previously to inhibit channel catfish neutrophil adhesion (Ainsworth et al., 1996). In this study RGD did not block poly IC- induced HA. Although ICAM-1 does not contain an RGD motif (Simmons et al., 1988), LFA-1 appears to be able to bind proteins containing RGD sequence (Gadek et al., 2002). The results in this study can be interpreted two ways, either LFA-1 is not involved in poly IC-induced HA in RTS11, or fish LFA-1 is different from human LFA-1 and does not bind to RGD. An LFA-1 specific inhibitor was also tried and did not block poly IC-induced HA in RTS11; however, this inhibitor has only been tested on mammalian cells and may not affect fish LFA-1.

Another possibility is that galectins, as opposed to integrins, are the adhesion molecule involved in poly IC-induced HA. Galectins are β-galactoside binding proteins involved in many cell responses including aggregation. Eosinophil aggregation *via* galectin-9 was found to be inhibited with the addition of EDTA, suggesting divalent cation dependence similar to that observed with integrins (Hirashima et al., 2004). Galectin expression is upregulated in human monocytes in response to LPS (Almkvist and Karlsson, 2004) and PMA (Kim et al., 2003). Poly IC induced increased galectin expression in human endothelials cells, and this upregulation was found to be mediated *via* PKR (Imaizumi et al., 2004). In fish it was shown that viral hemorrhagic septicemia virus (VHSV) induced expression of a galectin-9-like molecule in rainbow trout leukocytes (O'Farrell et al., 2002). Thus, it is possible that galectins are being upregulated in activated RTS11 in response to all stimuli tested, and more specifically in the case of poly IC, this galectin expression may be mediated *via* PKR.

PKR involvement

This study shows a direct role for PKR in controlling HA in RTS11. Two PKR inhibitors, 2-aminopurine (2-AP) and compound 16, were both able to block poly IC-induced HA in RTS11. In mammals, PKR has been reported to control expression of a variety of adhesion molecules. As mentioned above, PKR can mediate poly IC-induced galectin expression. Offerman *et al.* showed that poly IC-induced PKR activity resulted in increased levels of VCAM-1 in endothelial cells (Offermann et al., 1995). Poly IC has also been shown to induce E-selectin *via* a PKR dependent mechanism (Bandyopadhyayk et al., 2000). In our results, 2-AP completely blocked HA, while compound 16 only partially blocked poly IC-induced HA. The inhibitory actions of 2-AP may

not be completely specific to PKR and may affect the activity of other serine/threonine kinases (Essbauer and Ahne, 2002), while compound 16 has been developed more recently and is more specific to PKR alone (Jammi et al., 2003). Therefore it can be concluded that although PKR plays an important role in poly IC-induced HA, it is most likely not the only kinase involved.

It has been previously shown that poly IC treatment results in eIF2α phosphorylation in RTS11 (chapter 4). Although never directly tested in RTS11, mammalian literature suggests that eIF2α phosphorylation leads to global translation inhibition within the cell. Seemingly opposed to this, the current study shows that *de novo* protein synthesis is required for homotypic aggregation. Other studies have suggested that when eIF2α phosphorylation occurs, protein synthesis inhibition is not absolute and that sustained, high levels of selected mRNA transcripts are necessary to increase the likelihood of translation during this suppressed protein synthesis environment. VCAM-1 is one such transcript that is expressed at high levels for long periods of time in poly IC treated endothelial cells (Offermann et al., 1995). Thus, it is possible that the mRNA transcripts for the adhesion molecules responsible for HA in RTS11 are expressed at very high levels and are able to be expressed even with global translation inhibition.

Conclusions

RTS11 was found to undergo HA when responding to immunological stimuli, thus making HA a novel marker for general cell activation in RTS11. This is the first study of HA in fish cells that implicate roles for PKR and galectins. Further research is necessary to clarify the pathways involved in this mechanism; however, this research may prove important for understanding monocyte/macrophage activity during viral infections *in vivo*.

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Chapter 6

Cytopathic effects of chum salmon reovirus in salmonid epithelial, fibroblast and macrophage cell lines

ABSTRACT

Chum salmon reovirus (CSV) belongs to the aquareoviridae (AqRV) family of viruses, which has an icosohedral, double capsid, and an eleven segment dsRNA genome. These viruses also cause a characteristic cytopathic effect (CPE): syncytia or multinucleated giant cells. Syncytia formation has usually been studied with epithelial and fibroblast cell lines, but the responses of macrophages are of interest because they might be targets of AqRV. To this end, the CPE elicited by CSV has been compared in three salmonid cell lines: epithelial-like CHSE-214 from Chinook salmon embryo, fibroblast-like RTG-2, and monocyte/macrophage-like RTS11, both from rainbow trout. CHSE-214 supported the most substantial production of CSV and demonstrated the most dramatic syncytia formation. At 4 days of CSV exposure, the formation of syncytia was well advanced with CHSE-214, just beginning with RTG-2, and absent with RTS11, which instead underwent homotypic aggregation (HA). Syncytia and HA formation were blocked by cycloheximide and ribavirin but not actinomycin D. These results suggest that expression of CSV genes were required for both phenomena. Cultures with syncytia underwent a decline in cell viability, as measured using two fluorescent indicator dyes, alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester, which measure metabolic activity and membrane integrity respectively. At 4 days the decline in viability was more pronounced in CHSE-214 than in RTG-2 cultures. By 7 days of exposure to high CSV titres, cell viability in cultures of both cell lines had declined over 90 % and large syncytia were peeling off the plastic growth surface, especially in CHSE-214 cultures. As revealed by agarose gel electrophoresis, genomic DNA fragmentation was evident after 4 days in CHSE-214 and 7 days in RTG-2. In the presence of the pan-caspase inhibitor, zVAD-fmk, CHSE-214 cultures continued to form syncytia and show diminished energy metabolism, but DNA fragmentation, the loss of membrane integrity, and the release of infectious CSV were considerably blocked. These results suggest that the formation of syncytia triggers apoptosis and a leaky plasma membrane, which enhances viral release. By contrast, RTS11 cultures undergoing HA showed no loss of cell viability. The significance of HA is unclear, but the response suggests that macrophage behaviour in rainbow trout potentially could be modulated by CSV.

6.1. INTRODUCTION

Aquareoviruses (AqRV) have been isolated from many different fish species; however, their impact on fish health seems to vary with viral strain and host species. All AqRV are non-enveloped with an icosohedral, double capsid and an eleven segment dsRNA genome (Lupiani et al., 1995; Winton et al., 1981). Some AqRV, such as channel catfish reovirus (CCRV), cause economic losses in fish farms (Amend et al., 1984) and a few others are quite pathogenic (Seng et al., 2002). By contrast, the source of several AqRV from salmonids has been apparently healthy fish (Essbauer and Ahne, 2001). The first AqRV identified from salmonids was Chum salmon reovirus (CSV), which was isolated from Chum salmon being routinely tested at a Japanese aquaculture facility prior to the exportation of eggs (Winton et al., 1981; Winton et al., 1981). Despite being cytolytic to some salmonid cell lines, CSV caused little or no pathology to five salmonid species (Winton et al., 1989).

AqRV cause a characteristic cytopathic effect (CPE) (Lupiani et al., 1995). They induce the formation of syncytia, which are large multinucleated giant cells formed by the fusion of neighbouring cells. This unusual property among nonenveloped viruses is also found among many orthoreoviruses (ORV). The genus *Orthoreovirus* contains mammalian orthoreoviruses (MRV) including Nelson Bay orthoreovirus (NBV) and baboon orthoreovirus (BRV), avian orthoreoviruses (ARV), and reptilian orthoreoviruses (RRV) (Duncan, 1999). Typically, ARV and RRV are fusogenic; however, MRV are generally not fusogenic, with the exception of NBV and BRV. For ARV, syncytia formation enhances but is not essential for cytolysis and virus egress (Duncan et al., 1996). The viral proteins responsible for the induction of syncytia are the fusion-associated small transmembrane (FAST) proteins (Shmulevitz and Duncan, 2000). Recently, the induction of syncytia by FAST proteins has been shown to trigger apoptosis (Salsman et al., 2005). MRV also induce apoptosis, although without syncytia formation (Clarke et al., 2005). In contrast to ORV, little is known about the cell biology of AqRV infection, such as the occurrence and importance of apoptosis.

Nearly all studies on AqRV have used fish epithelial-or fibroblast-like cell lines (Lupiani et al., 1995), and very little is known of immune cell responses, particularly macrophages. For ARV, several studies indicate preferential replication in macrophages (Von Bulow and Klasen, 1983; O'Hara et al., 2001; Swanson et al., 2001) and the suggestion has been made that the mobility of these cells contributes to the dissemination of the virus and the pathological consequences to chickens (Kibenge et al., 1985). For AqRV, several isolations have been from fish concurrently infected with bacteria (Lupiani et al., 1989; Cusack et al., 2001), which possibly

indicates immunosuppression. In the case of turbot aquareovirus (TRV), primary macrophage cultures supported viral replication and were able to kill bacteria despite being infected with the virus (Rivas et al., 1996). This appears to be the only study of AqRV and macrophages. However, over the last few years several fish macrophage cell lines have become available (Bols et al., 2003), with one being the monocyte/macrophage cell line, RTS11 (Ganassin and Bols, 1998), enabling *in vitro* studies of viral/macrophage interactions.

In this chapter, the CPE of CSV has been compared in three cell lines: the epithelial-like CHSE-214 from Chinook salmon embryo, the fibroblast-like RTG-2 from the rainbow trout gonad, and RTS11 from the rainbow trout spleen. CSV caused syncytia to form in cultures of CHSE-214 and RTG-2 and homotypic aggregation (HA) in cultures of RTS11. CSV gene expression was required for both types of responses. Cultures displaying syncytia died by apoptosis, which enhanced the release of CSV. By contrast, HA did not lead to the loss of cell viability but did support a small amount of CSV production.

6.2. MATERIALS AND METHODS

6.2.1. Cell Culture and inhibitor treatments

Three cell lines were used in the course of this study: fibroblast-like RTG-2, monocyte/macrophage RTS11 and epithelial-like CHSE-214. RTG-2 and CHSE-214 were obtained from the American Type Culture Collection (ATCC), while RTS11 was developed in this laboratory (Ganassin and Bols, 1998). Cell lines were grown as previously described (DeWitte-Orr et al., 2005; DeWitte-Orr and Bols, 2005). Briefly, all cell lines were grown in L-15 medium supplemented with 150U/mL penicillin G, 150μg/mL streptomycin sulfate, and fetal bovine serum (FBS), 10% for RTG-2 and CHSE-214, and 15% for RTS11 (Sigma, St Louis, MO). The growing temperature for RTG-2 and CHSE-214 was 21^{o}C, while RTS11 was grown at 18^{o}C.

6.2.2. Virus propagation and infection

Chum salmon reovirus (CSV) was obtained from ATCC and routinely propagated on CHSE-214 monolayers. CSV containing media (CCM) was collected 7d post-infection (pi), passed through a 0.2 μm filter, and kept frozen at -80^{o}C until used. Virus titration was performed on monolayers of CHSE-214 cells grown in a 96-well plate. Viral suspensions were diluted from 10^{-1} to 10^{-6} and 6 wells were inoculated with 200 μL of each dilution. Cultures were incubated at 21 °C for 3 days. Following this period, the cell monolayers were scored for the appearance of cytopathic effects and the final titre, expressed as $TCID_{50}$ /mL, was estimated using the Karber method (Karber, 1931).

For all experiments, RTG-2 and CHSE-214 were grown to 90% confluency in 25cm^2 tissue culture treated flasks (Nunc, Roskilde, Denmark), and the medium was changed to 5% FBS immediately prior to virus infection. As RTS11 is a semi-adherent cell line and does not form a confluent monolayer, cells were plated at $1.0x10^7$ cells per 12.5 cm^2 tissue culture treated flask (Nunc) in L-15 supplemented with 5% FBS. In all experiments, cells were infected with serial dilutions of CCM, which had a viral titre of $10^{5.3}$ tissue culture infectious dose $(TCID)_{50}$/mL. The duration of infection for each cell line differed, based on development of cytopathic effect (CPE).

6.2.3. Inhibitor treatments

For the syncytia quantification and homotypic aggregation inhibition experiments, the cells were treated with specific inhibitors, ribavirin (100 μg/mL), actinomycin D (0.5 μg/mL), cycloheximide (0.1 μg/mL), zVAD-fmk (50 μM) and two dsRNA dependent protein kinase (PKR) inhibitors, 2-aminopurine (1.5mM) and a commercially available oxindole functionalized

with an imidazole, referred to as compound 16 (Jammi et al., 2003)(0.5 μg/mL), 30 minutes prior to virus exposure. The inhibitors were kept in the media with the virus for the duration of the experiment. Ribavirin, actinomycin D, cycloheximide and 2-aminopurine were purchased from Sigma; compound 16 (cat. no. 527450) and zVAD-fmk were purchased from Calbiochem (La Jolla, CA).

6.2.4. Viral replication assay

CSV was added to the three cell lines (10^{-1} diluted CCM) for 2h to allow for viral attachment. The medium was then removed, the cultures were washed twice with PBS (Sigma) and fresh medium was added to each culture, supplemented with 5% FBS. As RTS11 cultures are semi-adherent, viral exposures were terminated slightly differently. The cells were collected, centrifuged and washed twice with PBS in a 15mL polypropylene centrifuge tube (Falcon/Becton-Dickenson, Franklin Lakes, NJ), and plated in new tissue culture treated flasks with fresh media. These cultures were incubated for 4d for CHSE-214 or 7d and 12d for RTG-2 and RTS11. The media was then collected, passed through a 0.2μm filter, put on a fresh monolayer of CHSE-214 or RTG-2, serially diluted from 10^{-1} to 10^{-6}, and incubated for 3d. Syncytia formation was observed and a $TCID_{50}$ value was determined based on the Karber method. When studying the effects of zVAD-fmk on viral replication and release, cells were exposed to CSV similar to the replication assay; however, 50μM zVAD-fmk was added to the fresh media and cells were allowed to incubate for 4d for CHSE-214 and 12d for RTG-2 before the media was removed, filtered, diluted, and added to new CHSE-214 cultures. A $TCID_{50}$ value was determined 3d pi from the new CHSE-214 cultures.

6.2.5. Cell viability assay

Plating and viral exposure

Cells were seeded into 96 well plates (Falcon), in L-15 growing medium with the normal growing FBS supplement. CHSE-214 and RTG-2 cells were seeded at $3.0x10^4$ cells per well while RTS11 were seeded at $1.25x10^5$ cells per well. Cells were allowed to settle and reattach for 24 h at their normal growing temperature before being exposed the virus. The normal growth medium was removed, and CCM that was serially diluted (10^{-1}-10^{-6}) in L-15 supplemented with 5% FBS, was added to each culture.

Measuring cytotoxicity

As described previously, two fluorescent indicator dyes, alamar Blue™ (Medicorp, Montreal, PQ) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Molecular

Probes, Eugene, OR) were used to evaluate cell viability (DeWitte-Orr et al., 2005; DeWitte-Orr and Bols, 2005). Briefly, alamar Blue measures cellular metabolism (resorufin) (O'Brien et al., 2000), while CFDA-AM measures intracellular esterase activity, thus confirming membrane integrity (Schirmer et al., 1997). The levels of fluorescence are measured using a microplate spectrofluorometer. Alamar Blue and CFDA-AM assays were performed as previously described (Ganassin et al., 2000). Briefly, virus exposed cells were measured for cell viability at 4 and 7d pi. The media was removed and the two fluorescent dyes were added to the wells, diluted in a minimal salt solution called L-15ex. The cells were incubated with the dyes for 60 minutes before the microwells were read with a fluorometric plate reader (Molecular Devices SpectraMax). Excitation and emission wavelengths were 530 and 595 nm for alamar Blue and 485 and 530 nm for CFDA-AM.

6.2.6. Genomic DNA (gDNA) laddering assay for apoptosis

Cells were exposed to 10^{-1} diluted CCM for the specified length of time. After which, the cells were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit (Sigma), which is based on column affinity technology. The genomic DNA was eluted from the column using 100 µL Milli-Q water, and 35 µL of this DNA was resolved by electrophoresis on a 2% (w/v) agarose gel for 4 hours at 60V. The DNA ladders were visualized by staining gels with 0.5 µg/mL ethidium bromide (EtBr) and photographed under a UV transillumination.

6.2.7. Cytochemical staining of syncytia

CSV exposed cultures were stained to visualize syncytia formation or the lack thereof, using a Romanowsky-Giemsa stain. Cells were seeded into $9cm^2$ slide flasks (Nunc) at $8.4x10^5$ cells per flask for RTG-2 and CHSE-214, and $3.5x10^6$ cells per flask for RTS11. Cells were exposed to the virus and inhibitors as described in sections 6.2.2 and 6.2.3. At the specified time point, cells were fixed in anhydrous methanol for 10 minutes, washed in distilled water for 1 min and allowed to air dry. The slides were then submerged in stain (3 mg/mL azure B bromide in DMSO, 1 mg/mL eosin Y in anhydrous methanol, diluted 1:15 in 10mM HEPES buffer [10mM HEPES, 5% DMSO, pH 6.8]) for 30 minutes. Azure B bromide was purchased from Manufacturing Chemists (Norwood, OH), and eosin Y was purchased from Allied Chemical (Morriston, NJ). Slides were then rinsed in distilled water for 1 minute, and allowed to air dry. Slides were mounted using a non-aqueous mounting medium and visualized using a Nikon Optiphot microscope (Japan).

6.2.8. Syncytia quantification

CHSE-214 were seeded into 60mm X 15 mm tissue culture treated Petri plates (Corning, Corning, NY) at $1.0x10^6$ cells per plate. Cells were allowed to attach overnight and were then exposed to CSV and inhibitors as described in sections 6.2.2 and 6.2.3. For syncytia quantification experiments, syncytia were counted from cultures treated with CCM diluted 10^{-3}. After 3d the cells were fixed and stained using Romanowsky-Giemsa stain as described in section 6.2.7. Digital pictures were taken of four random fields of view, at 40X magnification. Total number of syncytia were counted from each picture and averaged. Each experiment was repeated at least three times. Results were reported as a percent of the number of syncytia counted from cultures exposed to CSV alone.

6.2.9. Homotypic aggregation quantification

Homotypic aggregation was quantified as previously described (chapter 5). Briefly, RTS11 cells were seeded in 96-well plates at $1.25x10^5$ cells per well and allowed to attach overnight. Cells were then exposed to serially diluted CSV-containing conditioned media and inhibitors as described in sections 6.2.2 and 6.2.3. Cells were examined for homotypic cell aggregation 4d pi, using a phase-contrast microscope. Representative pictures of each well were taken at 200X magnification; therefore 6 pictures were taken per treatment. Using Adobe Photoshop, aggregates larger than a 0.04 mm by 0.04 mm square were counted. In general, this particular size marker would include between 15 to 25 cells per aggregate. Any aggregate that did not fill the square was not included in the count.

6.2.10. Data analysis

For alamar Blue and CFDA-AM assays, cytotoxicity was indicated by a decline in fluorescence units (FUs) for experimental cultures relative to control cultures. FUs for culture wells with no cells were constant and subtracted from FUs for experimental and control cultures. For graphical presentation, the results were plotted using SigmaPlot (Jandel Scientific). For the viral release assay, $TCID_{50}$ values were compared using a one-way ANOVA with a Student-Newman-Keuls multiple comparisons post test ($p \leq 0.05$). All statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com).

6.3. RESULTS

6.3.1. CSV cytopathic effects (CPE)

CSV cytopathic effects (CPE) proved different in the three teleost cell lines tested: RTS11, RTG-2 and CHSE-214. RTS11 underwent homotypic aggregation, a cellular adhesion of like cell type typical of activated immune cells; however, not until 4d post-infection (pi;Figure 6-1). RTG-2 and CHSE-214 both formed syncytia; however, the morphology and timing of the syncytia were different (Figure 6-1). In CHSE-214 cultures, syncytia began to form by 2d pi, grew larger over the next few days, and began to lift off the growth surface by 4d pi. Conversely, RTG-2 formed very few syncytia, and only after 4d pi. These syncytia were much smaller in size, had a more defined periphery, often contained a ring of nuclei in the centre, and appeared to detach from the growth surface differently. CHSE-214 syncytia appeared to detach as sheets that often rolled back over the remaining monolayer, whereas RTG-2 syncytia lifted off the surface as intact 'balls' (Figure 6-1).

The mechanism of CSV-induced syncytia formation in CHSE-214 was investigated with various inhibitors (Figure 6-2A&B). Ribavirin, an inhibitor of viral transcription, and cycloheximide (CHX), a translational inhibitor, blocked the formation of syncytia. Syncytia formed in the presence of actinomycin D (AMD), a transcriptional inhibitor, zVAD-fmk, a pan-caspase inhibitor (Figure 6-5), and either 2-aminopurine (2-AP) or compound 16, both inhibitors of dsRNA-dependent protein kinase, PKR (Figure 6-2A&B).

6.3.2. CSV replication in three salmonid cell lines

CSV replication was supported by all three cells lines, CHSE-214, RTG-2, and RTS11 (Table 6-1). Infectious virions were evident in the extracellular medium for cultures of CHSE-214 by 4 d, but not until 12 d for RTG-2 and RTS11. At 4d pi for CHSE-214 and 7 and 12 days pi for RTG-2 and RTS11, cells were disrupted by freeze-thaw cycles to examine for the presence of CSV in the intracellular compartment. Intracellular infectious particles could be detected in CHSE-214 at 4d pi, and at 12d pi for both RTS11 and RTG-2. Infectious particles could not be detected intracellularly in 7d pi cultures of RTS11, which were assayed on CHSE-214, nor of RTG-2, which were assayed on both CHSE-214 and RTG-2. Therefore, CSV replication appears much slower in RTG-2 and RTS11 compared to CHSE-214.

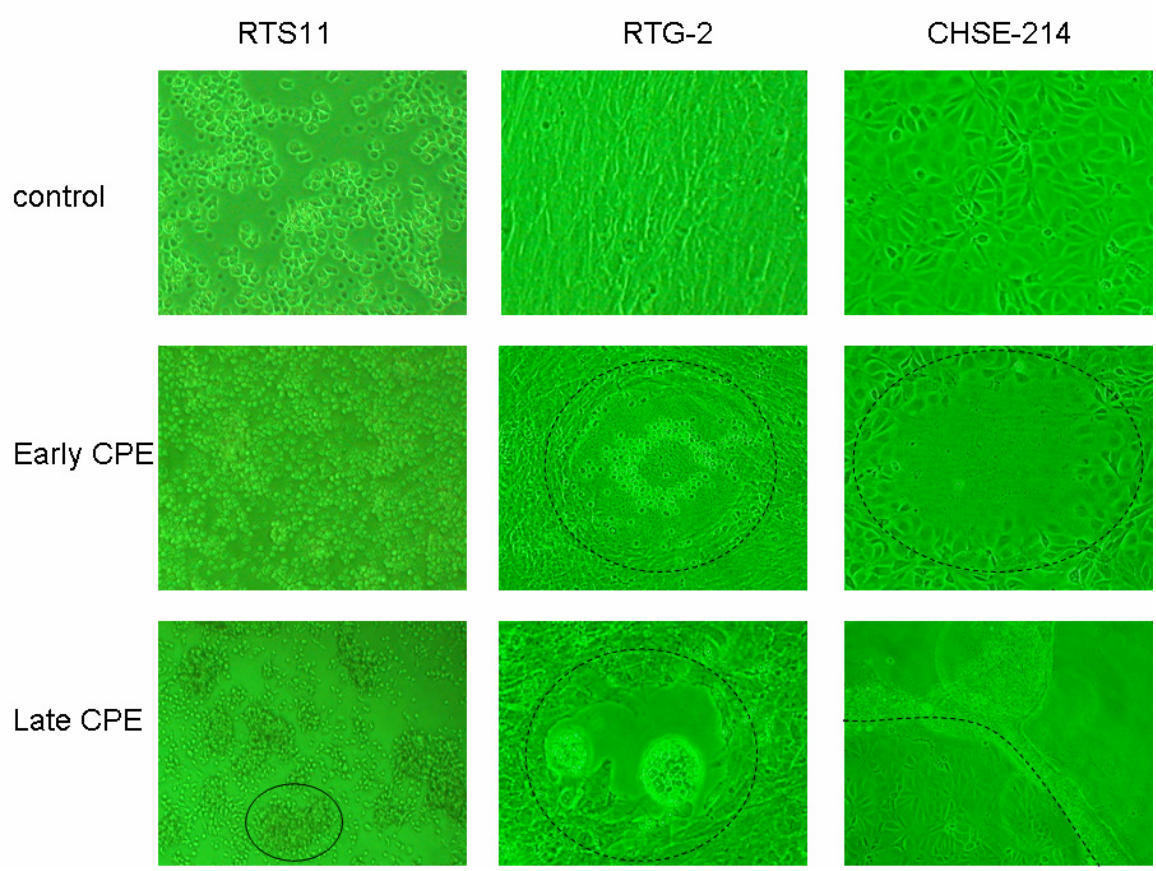

Figure 6-1. Cytopathic effect (CPE) of CSV on three salmonid cell lines.

The top row shows the phase-contrast microscopy appearance of high density cultures of the macrophage/monocyte cell line, RTS11, fibroblast cell line, RTG-2 and the epithelial cell line, CHSE-214. The middle and bottom rows show the appearance of the same cultures demonstrating early and late CPE following the addition of CSV. Early CPE were observed at 2d for CHSE and 4d for RTG-2 and RTS11, while late CPE were observed at 4d for CHSE-214 and 7d for RTG-2 and RTS11. Areas of homotypic aggregation are identified by solid lines and syncytia formation by dashed lines. Magnification is 100X.

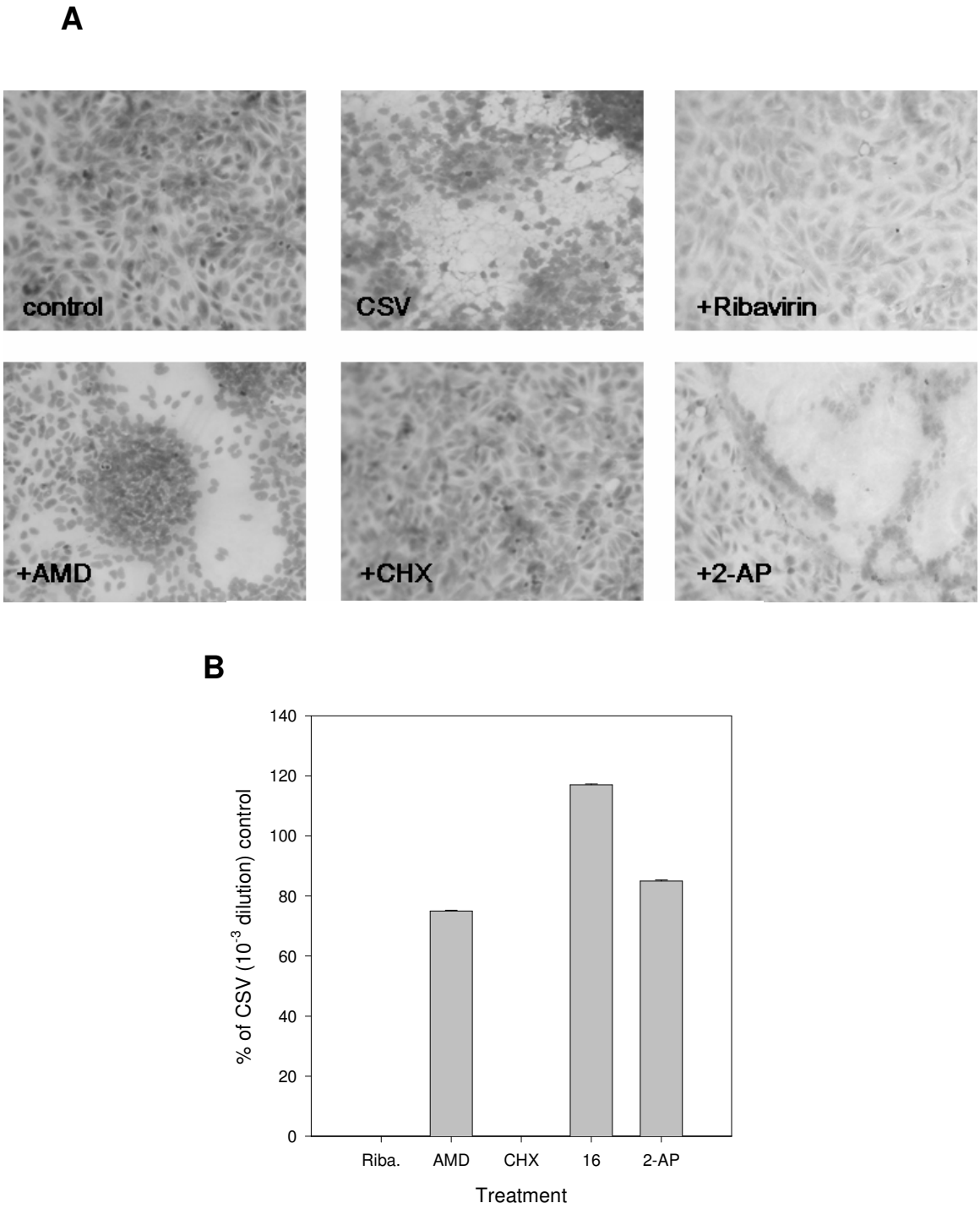

Figure 6-2. Inhibition of CSV-induced syncytia in CHSE-214.

CHSE-214 monolayers were treated with 100µg/mL ribavirin, 0.5µg/mL actinomycin D (AMD), 0.1µg/mL cyclohexmide (CHX), 1.5mM 2-aminopurine (2-AP) or 0.5µg/mL compound 16 (16) for 30 minutes prior to co-exposures with 10^{-3} diluted CCM. After 3d, the cultures were fixed, stained with Romanowsky-Giemsa stain, and syncytia quantified. **A** Representative pictures of control and treated cultures. Magnification is 100X **B** Quantified syncytia from treated cultures, plotted as a % of CSV alone control. The results are representative of three separate experiments.

Table 6-1. Production of infectious Chum salmon reovirus (CSV) on three different salmonid cell lines.

Conditions for CSV production			Reporting infectious CSV production		
cell line	1 h viral exposure*	Time to condition medium (CM)	cell line♠	Time in CM	$TCID_{50}$♣ (/mL)
CHSE-214	None (control)	4 days	CHSE-214	3 days	-
	CCM	4 days	CHSE-214	3 days	$10^{5.0}$
	intracellular	4 days	CHSE-214	3 days	$10^{3.6}$
RTG-2	None (control)	7 days	CHSE-214	3 days	-
	CCM	7 days	CHSE-214	3 days	-
	intracellular	7 days	CHSE-214	3 days	-
	CCM	7 days	RTG-2	7 days	-
	CCM	12 days	CHSE-214	3 days	$10^{2.5}$
	intracellular	12 days	CHSE-214	3 days	$10^{2.8}$
RTS11	None (control)	7 days	CHSE-214	3 days	-
	CCM	7 days	CHSE-214	3 days	-
	intracellular	7 days	CHSE-214	3 days	-
	CCM	12 days	CHSE-214	3 days	$10^{0.8}$
	intracellular	12 days	CHSE-214	3 days	$10^{1.5}$

*Cultures at 18 °C were exposed to serial dilutions of CSV containing media (CCM, with $TCID_{50}=10^{5.3}$/mL) for 2 h at which time the medium removed, cultures washed, and fresh medium without virus was added to be conditioned.

♠ The conditioned medium was serially diluted, applied to new cultures, and monitored for CPE. Intracellular infectious particles were measured by lysing the cells and applying the cell lysate to new cultures to monitor for CPE.

♣Syncytia formation was quantified by determining a $TCID_{50}$ value, indicating the production of infectious CSV.

6.3.3. CSV induced cytotoxicity *via* apoptosis

CSV exposure killed RTG-2 and CHSE-214 in a viral titre dependent manner as measured using fluorescent indicator dyes, alamar Blue and CFDA-AM (Figure 6-3). This cell death was time dependent, as more cytotoxicity could be detected at 7d pi compared with 4d pi. Cytotoxicity could not be detected in CSV exposed RTS11 cultures, even after 7d pi. In fact, RTS11 cultures demonstrated increased metabolic activity at high viral titres (Figure 6-3).

The CSV-induced death of RTG-2 and CHSE-214 appeared to be *via* apoptosis. Fragmentation of genomic DNA (gDNA) into 180 bp oligomers, a hallmark of apoptosis, was detected in virus-treated CHSE-214 and RTG-2 but not in RTS11 cultures (Figure 6-4). The DNA laddering was inhibited by CHX (Figure 6-4) and ribavirin (data not shown), both of which blocked syncytia formation (Figure 6-2), and by zVAD-fmk (Figure 6-6B), which did not inhibit the development of syncytia (Figure 6-5). Thus, CSV-induced DNA laddering required the formation of syncytia first and then proceeded by a caspase-mediated process.

6.3.4. Cell membrane integrity and virus release

Upon treatment of RTG-2 and CHSE-214 with CSV, a diminishment in energy metabolism as measured with alamar Blue appeared to occur concurrently with a loss in cell membrane integrity as measured with CFDA-AM. This was evident in CHSE-214 cultures at 4 d pi and 7d pi (Figure 6-3). The loss of membrane integrity was delayed by zVAD-fmk, but ultimately did occur (Figure 6-6A). The phase contrast microscopy appearance of CSV-treated cultures was unaltered in early infections by zVAD-fmk. However, in late infections, rather than lifting and rolling off the culture surface, the syncytia remained attached to the growth surface, although they seemed disrupted (Figure 6-5). Thus, as well as dying by apoptosis, the syncytia possibly can die by an alternate mechanism when caspases are inhibited.

The release of CSV was only moderately influenced by zVAD-fmk and only with CHSE-214 (Figure 6-7). For CHSE-214, intracellular virion counts remained unchanged with zVAD-fmk exposure; however, zVAD-fmk did decrease extracellular virion levels. For RTG-2, zVAD-fmk did not affect viral particle counts in either the intracellular or extracellular environments.

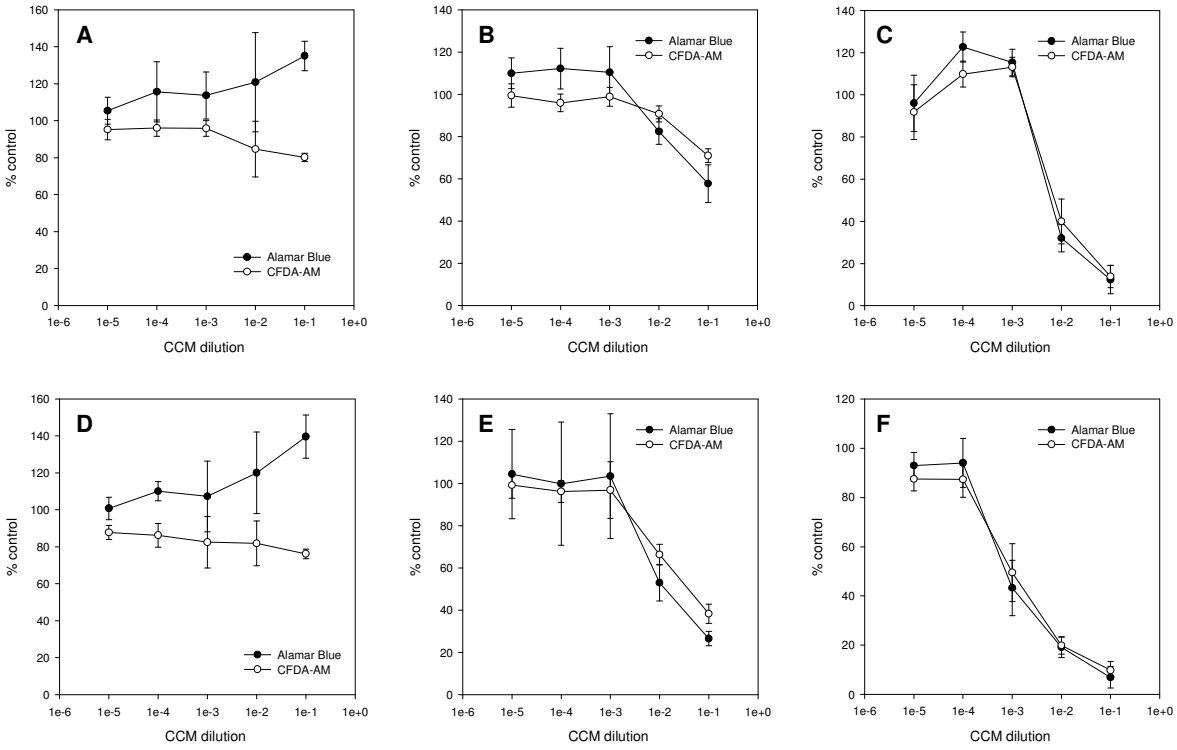

Figure 6-3. Effect of CSV on the viability of three salmonid cell line lines.

Cultures of RTS11 (**A** & **D**), RTG-2 (**B** & **E**) and CHSE-214 (**C** & **F**) in L-15 with 5% FBS at 18 °C were either untreated controls or exposed to varying serial dilutions of CCM. The x-axis shows the serial dilution of CCM on a logarithmic scale. The y-axis represents cell viability, which was expressed as a % of control cultures and was measured 4 days (**A**, **B** & **C**) and 7 days (**D**, **E** & **F**) after the start of CSV exposure. Cell viability was measured in each culture with alamar Blue, which is a measure of energy metabolism, and CFDA-AM, which provides an indication of plasma membrane integrity. The results are representative of three separate experiments.

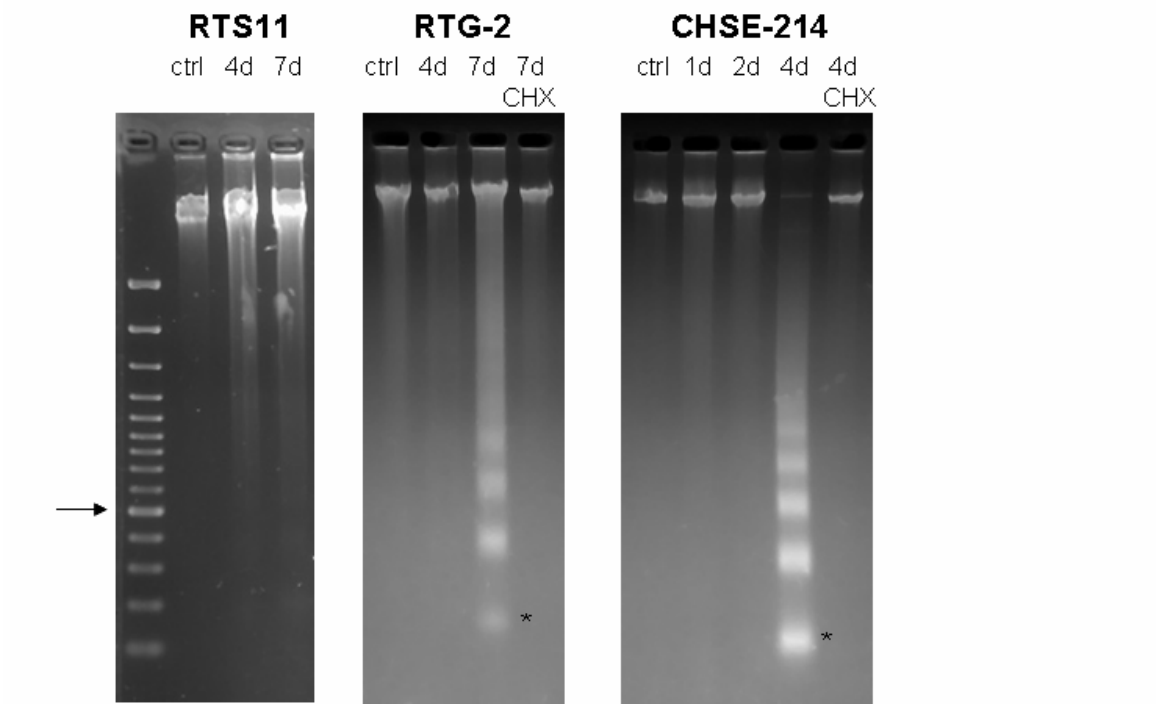

Figure 6-4. Effect of CSV on the integrity of genomic DNA in three salmonid cell lines.

Cultures of RTS11, RTG-2 and CHSE-214 in L-15 with 5% FBS at 18 °C were either untreated controls (ctrl) or exposed to 10^{-1} diluted CCM. As well as receiving CSV, some RTG-2 and CHSE-214 cultures were dosed additionally with the translational inhibitor, cycloheximide (CHX, 0.1 μg/mL). At various times afterwards, genomic DNA was extracted from cultures and run on 2% agarose gels. Above each gel lane is an indication of the culture treatment and/or time in days (d) at which genomic DNA was extracted. The lane at the far left is a DNA marker with 100bp increments and an arrow at 500bp. Asterisks identify the positions of 180 bp fragments. The results are representative of two separate experiments.

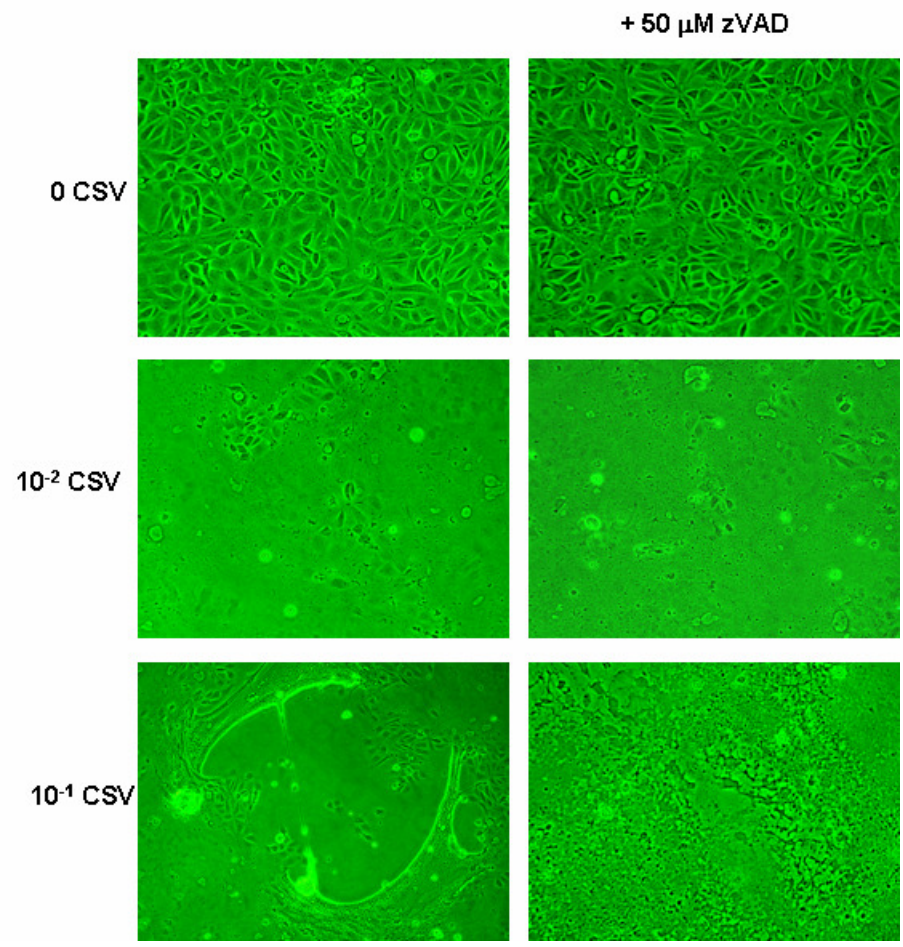

Figure 6-5. The effect of zVAD-fmk, a pan-caspase inhibitor, on CHSE-214 culture morphology following exposure to CSV.

CHSE-214 monolayers were treated with serially diluted CCM (10^{-1}, 10^{-2} dilutions), with or without co-treatments of 50 µM zVAD-fmk (zVAD). Changes in culture morphology were recorded 4d pi (100X magnification). The results are representative of three separate experiments

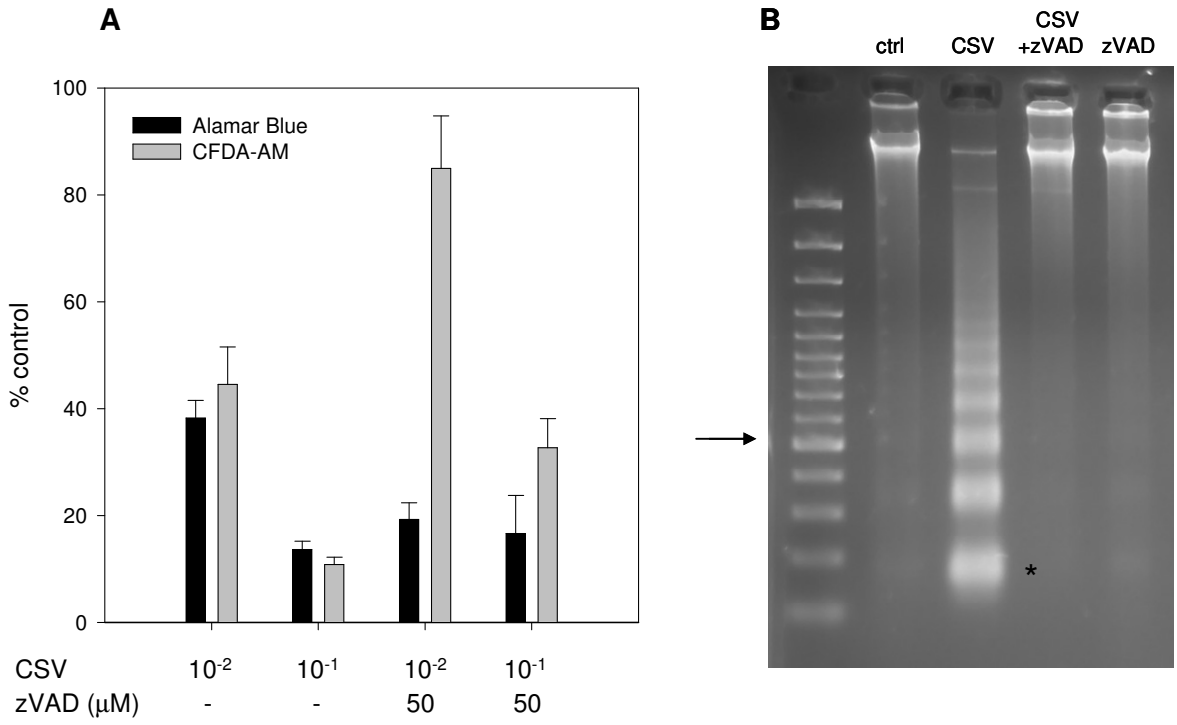

Figure 6-6. The effects of zVAD-fmk on the CPE of CSV to CHSE-214.

Cultures of CHSE-214 in L-15 with 5 % FBS at 18°C were exposed to serially diluted CCM (10^{-1} and 10^{-2}), to the caspase inhibitor, zVAD-fmk (zVAD, 50 μM), or to the two together (CSV + zVAD). After 4 days, cultures were examined for their cell viability (**A**), and genomic DNA (gDNA) integrity (**B**). CCM diluted 10^{-1} was used for the gDNA laddering experiment. Cell viability was assessed with alamar Blue and CFDA-AM as described in Figure 6-3 and genomic DNA was analyzed as described in Figure 6-4, with the asterisk identifying the position of 180 bp fragment and the arrow pointing to 500 bp in the lane of DNA markers. The results are representative of two separate experiments.

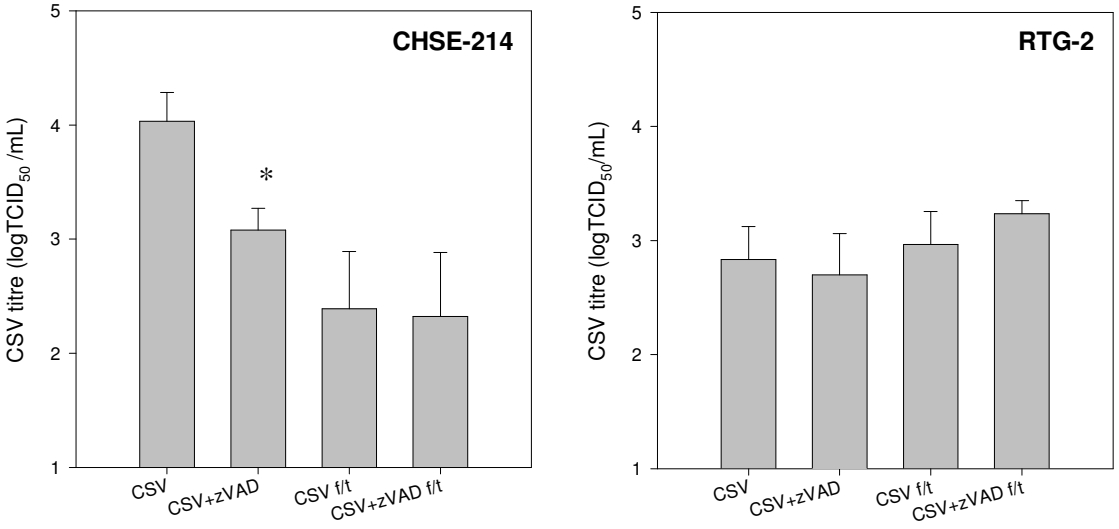

Figure 6-7. Effect of zVAD-fmk on CSV release in CHSE-214 and RTG-2.

Confluent monolayers of CHSE-214 and RTG-2 were exposed to 10^{-1} diluted CCM with or without co-treatment with 50µM zVAD-fmk (zVAD). Following exposures of 4d for CHSE-214 and 12d for RTG-2, the number of virions were quantified from the extracellular media and intracellular space and calculated as a $TCID_{50}$ value. The intracellular viral particles were obtained by lysing the culture monolayer using three freeze/thaw cycles (f/t). For CHSE-214, the difference in $TCID_{50}$ values for extracellular virions from cultures treated with CSV+zVAD were found to be statistically significant when compared to CSV alone ($p<0.05$). There was no statistical significance for intracellular virions values between treatments, or between any treatments with RTG-2. These results are representative of three separate experiments.

6.3.5. CSV induced homotypic aggregation (HA) in RTS11

In RTS11, HA was evident 4d exposure to the virus (Figure 6-8A & B) and the magnitude of aggregation increased with the viral titre during the exposure (Figure 6-8 C). HA did not develop in the presence of CHX, demonstrating that CSV-induced aggregation required an active cell response and was not simply agglutination. HA was also blocked by ribavirin, but not by AMD or the PKR inhibitors (Figure 6-8D).

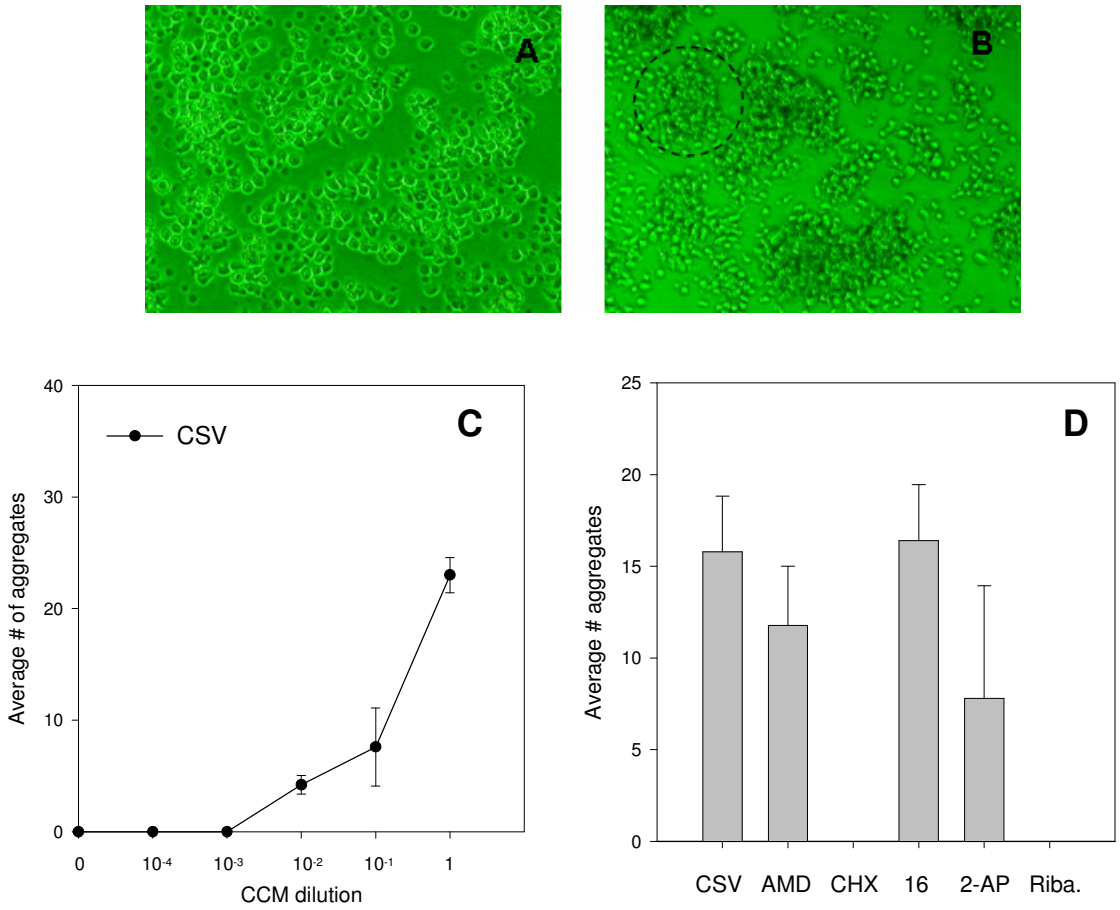

Figure 6-8. Induction of RTS11 HA by CSV and the inhibition of CSV-induced HA.

Representative pictures of control (**A**) and CSV treated (**B**) cultures, with an sample aggregate outlined by a dashed circle in the CSV treated culture. (100X magnification) **C** RTS11 at 18 ^{o}C were exposed to varying dilutions of CSV. The x-axis is logarithmic and shows the dilution of CCM. In both panels the y- axis is the average number of aggregates. **B** All cultures were exposed to CSV. The first bar shows the number of aggregates following CSV exposure in RTS11 at 18 ^{o}C and can be regarded as the control. The other bars show the number of aggregates in RTS11 cultures with CCM (10^{-1}) at 18 ^{o}C together with actinomycin D (AMD, 0.5 µg/mL), cycloheximide (CHX, 0.1 µg/mL), compound 16 (16, 0.5 µg/mL), 2-aminopurine (2-AP, 1.5 mM) or ribavirin (Riba., 100 µg/mL). All exposures were 4d in duration. The results are representative of 3 separate experiments.

6.4. DISCUSSION

Responses to Chum salmon reovirus (CSV) varied between cell lines. In the epithelial-like CHSE-214 cultures, syncytia formed within 3 days, became very large with time, and produced virus, which is in agreement with previous reports (Winton et al., 1981). In the fibroblast-like RTG-2 cultures, syncytia formed more slowly, were smaller, and produced virus only after long incubation periods. Previously, RTG-2 was found to produce low amounts of virus, but no syncytia formation was reported (Winton et al., 1981). As ARV-induced syncytia formation depends on pH (Duncan, 1996; Ni and Ramig, 1993), slight differences in culture conditions between the two studies could account for the differing capacities of RTG-2 to form syncytia in response to CSV. In monocyte/macrophage RTS11 cultures a novel cell response, homotypic aggregation (HA), was observed and very little virus was produced.

Induction of syncytia by CSV in epithelial and fibroblast cell lines

The expression of CSV gene(s) was required for syncytia formation in CHSE-214 and RTG-2 cultures, while host cell gene expression did not seem to be critical. Syncytia formation was blocked by ribavirin but not by actinomycin D (AMD). Ribavirin has been found to preferentially inhibit the transcription of viral genes for reoviruses in some host mammalian cells (Rankin et al., 1989). Consequently, it was presumed to act similarly with CSV and the salmonid cell lines used in this study. Also, for several mammalian reoviruses, AMD was shown not to impair viral RNA synthesis, although host cell RNA synthesis was inhibited (Shatkin, 1965; Stefanelli et al., 2002). In the current study, AMD was used at a concentration that almost completely blocked transcription in RTG-2 and CHSE-214 cultures (DeWitte-Orr et al., 2005). Translation of the viral transcripts was necessary for syncytia formation because cycloheximide (CHX) at a concentration that almost completely inhibited protein synthesis in RTG-2 and CHSE-214 (DeWitte-Orr et al., 2005) blocked the development of syncytia. The effects of these inhibitors of macromolecular synthesis on the induction of syncytia by AqRV have not been studied previously, but the responses seem similar to those of avian orthoreoviruses (ARV). The capacity of an ARV to induce syncytia in the monkey fibroblast cell line, VERO, was not impaired by AMD but was prevented by CHX (Ni and Ramig, 1993). In the case of ARV, the viral proteins responsible for syncytia formation were fusion-associated small transmembrane (FAST) proteins (Shmulevitz and Duncan, 2000; Salsman et al., 2005). Whether a FAST protein was involved in the syncytia arising from CSV infection is unknown but seems likely as a similar protein has been translated from one of the small AqRV segments (Kim et al., 2004).

Expression of several possible antiviral mechanisms in host cells appeared to have little effect on the formation of syncytia. For RTG-2, CSV was shown to induce transcripts of the antiviral proteins, Mx, in cultures without ribavirin, in which syncytia did form, and in cultures with ribavirin, in which syncytia formation did not occur (chapter 4). Apoptosis is considered an antiviral mechanism under some circumstances and is activated and executed by caspases (Clarke et al., 2005). In CHSE-214 and RTG-2 cultures, CSV continued to induce syncytia in the presence of the pan-caspase inhibitor, zVAD-fmk. The induction of syncytia by the FAST proteins of fusogenic orthoreoviruses (ORV) also occurred in the presence of zVAD-fmk (Salsman et al., 2005). Activation of dsRNA-dependent protein kinase (PKR) mediates the expression of several antiviral proteins, including interferons (Malmgaard, 2004); however, CSV was able to induce syncytia formation in CHSE-214 and RTG-2 cultures in the presence of PKR inhibitors. Interestingly, in mouse cells, PKR was shown recently not to interfere with reovirus replication, despite being activated by the virus (Smith et al., 2005).

Syncytial cell death

Once formed, the syncytia induced by CSV in CHSE-214 and RTG-2 cultures demonstrated a transitory life and lost viability. Membrane integrity and energy metabolism concurrently declined as syncytia began to dominate the culture. The loss of membrane integrity was seen as a decreased ability to metabolize 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) and to retain the fluorescent metabolite, CF (Schirmer et al., 1997). This is the first suggestion of membrane permeabilization in the CPE of AqRV, but such a step has been described for the fusogenic ORV, such as ARV. In the case ARV, membrane permeabilization was seen as the release of [^{3}H]-uridine and appeared after the development of syncytia (Salsman et al., 2005). Furthermore, transfection experiments showed that FAST proteins of four different ORV induced syncytia, followed later by the release of [^{3}H]-uridine (Salsman et al., 2005). By contrast, another study with ARV found that permeabilization preceded syncytia formation (Bodelon et al., 2002). In the current study with CSV, the loss of membrane integrity appeared to occur after the first appearance of syncytia, as CFDA-AM readings continued to decline between 4 and 7 days post-infection (pi), when the cultures became more overtaken by large syncytia (Figure 3) and not at early time points when syncytia were just beginning to develop (data not shown). The diminishment of energy metabolism was measured as a drop in rezasurin reduction (alamar Blue) (Schirmer et al., 1997; Abe et al., 2002) and occurred in parallel with the decline in CFDA-AM readings.

The death of syncytia-containing cultures was by apoptosis. Evidence for this was the cleavage of genomic DNA into oligonucleosomal sized fragments and the prevention of this damage by zVAD-fmk. This appears to be the first demonstration of apoptosis with AqRV, but previously apoptosis has been observed with ORV from mammals (Clarke et al., 2005) and birds (Labrada et al., 2002; Salsman et al., 2005). Apoptosis in CHSE-214 and RTG-2 cultures appeared to be triggered by the formation and maturation of syncytia as DNA fragmentation only became apparent in cultures in which most of the nuclei were in syncytia and in which membrane integrity had declined significantly. It is unclear whether the viral gene expression necessary for syncytia formation is sufficient for induction of apoptosis or whether further viral gene expression and/or host gene expression is needed as well. In the case of fusogenic ORV, the induction of syncytium formation by FAST proteins alone triggered apoptosis (Salsman et al., 2005). In contrast to these fusogenic reoviruses, some mammalian and avian reoviruses were able to induce apoptosis without viral gene expression (Connolly and Dermody, 2002; Labrada et al., 2002; Clarke and Tyler, 2003).

Apoptosis seems to be associated with loss of membrane integrity in CSV exposed CHSE-214 and RTG-2, as zVAD-fmk substantially inhibited the decline in CFDA-AM readings in cultures with mature syncytia. In a quail cell line, the triggering of apoptosis by FAST proteins also contributed to membrane permeabilization (Salsman et al., 2005). With the fish cell lines and CSV, energy metabolism continued to remain low despite the presence of zVAD-fmk. Possibly the impairment of energy metabolism is either a very early step in the programmed cell death, occurring prior to caspase activation, or an action of CSV independent of apoptosis.

Syncytial cell death and virus release

The apoptosis-associated loss of membrane integrity appeared to enhance virus release in CHSE-214. As evidence of this, under some conditions of virus infection, zVAD-fmk partially blocked the production of extracellular virus. Only virus release appeared to be affected and not virus replication as similar levels of infectious virions were obtained upon physically disrupting cells that had been treated with CSV with or without zVAD-fmk co-treatment. These results are similar to those observed with ARV (Duncan et al., 1996; Salsman et al., 2005). In the case of ARV, syncytium formation followed by apoptosis was thought to aid in systemic dissemination of the infection (Duncan et al., 1996; Salsman et al., 2005). It is important to note that as with our study, ARV release was not completely blocked with blocked apoptosis (Salsman et al., 2005), suggesting that apoptosis aids but is not essential for viral release. This is also an attractive idea for syncytium formation and apoptosis with CSV. Virus release in RTG-2 was

detected far later during the viral exposure compared to when apoptosis was first detected, 12d as opposed to 7d pi. This data, combined with the fact that zVAD-fmk did not affect viral release in this cell line suggests that CSV may exit RTG-2 using a mechanism different from the apoptosis associated loss of membrane integrity proposed in CHSE-214.

CSV and the monocyte/macrophage cell line, RTS11

CSV neither induced syncytia nor was cytolytic to RTS11. CSV did not diminish the membrane integrity of RTS11, but did appear to enhance energy metabolism, which was seen as in increase in the reduction of resazurin (alamar Blue). Currently, this is unexplained but under investigation. Interestingly, the turbot reovirus (TRV) appeared to affect the production of superoxide by turbot macrophages but did not induce syncytia nor cause cytolysis (Rivas et al., 1996).

CSV did cause the cells in RTS11 cultures to adhere to one another, which is referred to as homotypic aggregation (HA) and is a characteristic of activated immune cells (Frostegard et al., 1990). This cell response was deemed aggregation and not passive agglutination based on the requirement for *de novo* protein synthesis. These results appear to be the first evidence for reovirus-induced HA; however, HA has been shown to be induced by other viruses. In the paraymyxoviridae family of viruses, the measles virus caused aggregation of the human monocyte/macrophage cell line, U-937 (Soilu-Hanninen et al., 1996). As well, latent Epstein-Barr virus infections in B lymphocytes triggered HA (Park and Faller, 2002; Park and Faller, 2002) and the human T-cell leukemia virus type I (HTLV-1) Tax protein induced HA in human T cells (Takahashi et al., 2002; Takahashi et al., 2002). The role of HA in each of these cases is incompletely understood, although generally cell-to-cell contact regulates multiple cellular functions, such as leukocyte trafficking and activation, cytokine production and antigen presentation (Cho et al., 2003).

As with CSV-induced syncytia in CHSE-214 and RTG-2, CSV-induced HA in RTS11 was blocked by ribavirin and not by AMD, which suggests that HA required transcription of CSV gene(s) but did not necessarily require host transcript expression. The CSV transcripts would have to be translated as CHX blocked HA, but the nature of the proteins required for HA is unknown. As a viral protein found in the host cell membrane, it is possible that FAST proteins may have a role. Previously, artificial dsRNA was found to induce HA in RTS11 in a process mediated by dsRNA protein kinase (PKR) (chapter 5); however, the mechanism behind the HA induced by CSV appears to be different, as HA in RTS11 developed in the presence of PKR inhibitors.

CSV was able to establish a productive, albeit weak, infection in RTS11. By contrast avian macrophages were particularly susceptible to ARV (Von Bulow and Klasen, 1983; O'Hara et al., 2001). In the only previous study on an AqRV and macrophages, turbot reovirus (TRV) replicated in primary cultures of turbot macrophages (Rivas et al., 1996). As with CSV and RTS11, TRV did not induce the formation of syncytia or obvious cell death of the turbot macrophages (Rivas et al., 1996). Transmission electron microscopy revealed TRV release by budding from the macrophage plasma membrane (Rivas et al., 1998). This might be the case with CSV in RTS11 cultures as well. Though release of non-enveloped viruses is primarily through cell lysis, members of the *Orbivirus* genus, which belongs to the reoviridae family of viruses, were recently shown to be released from host cells by budding from the plasma membrane (Wirblich et al., 2006). The ability of CSV to induce HA in RTS11 suggests the potential for CSV to modulate macrophage functions. Also, the ability of RTS11 to support CSV production without obvious cytotoxicity suggests macrophages could aid in the dissemination of CSV within a fish.

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Chapter 7

Development of a novel continuous cell line, PBLE, from an American Eel peripheral blood leukocyte preparation

ABSTRACT

A continuous cell line, PBLE, was developed from the adherent cells in a culture of peripheral blood leukocytes from the American eel, *Anguilla rostrata*. Although two cell lines have previously been developed from the genus *Anguilla,* this represents the first cell line derived from *A. rostrata*. The cells were grown in Leibovitz's L-15 basal medium supplemented with 20 % fetal bovine serum (FBS). Under normal culture conditions, 18 °C, the morphology of PBLE were fibroblast-like. The cultures have been subcultivated over 80 times. PBLE have been cryopreserved successfully, have a diploid karyotype of 38 chromosomes, withstood temperatures from 5 to 36 °C, and proliferated at temperatures from room temperature to at least 30 °C. PBLE underwent apoptosis in response to gliotoxin, but did not show induction of 7-ethoxyresorufin o-deethylase (EROD) activity or a respiratory burst. Results suggest that PBLE may have arisen from a circulating mesenchymal stem cell. PBLE was susceptible to Chum salmon reovirus (CSV) and supported CSV replication. Therefore this cell line should be useful in studying eel specific viral-host interactions.

7.1. INTRODUCTION

The freshwater eels of the genus *Anguilla* Schrank are have long been subjects of scientific study because of their complex life history and their commercial value; however, in the last decade eel populations have declined dramatically, intensifying interest in this group of teleosts (Lecomte-Finiger, 2003; Stone, 2003). The population decline has been most studied for the only two species in the Atlantic Ocean, *Anguilla rostrata* and *A. anguilla* (Haro et al., 2000; Wirth and Bernatchez, 2003). The cause of the declining eel numbers is a matter of debate, but recently viral diseases have been suggested to play a role (van Ginneken et al., 2005). Unfortunately, cell lines to study eel diseases are few, with only two cell lines being derived from *Anguilla*, both from *A. japonica* (Chen and Kou, 1987; Fryer and Lannan, 1994).

Cell lines from fish are valuable for exploring many aspects of fish biology. They are particularly useful in detecting viruses (Fryer and Lannan, 1994) and in studying the molecular and cellular basis of physiological processes and toxicological mechanisms (Bols et al., 2005). Although a cell line from a fish species that is not of direct interest can be a useful surrogate, having lines from the species of interest is superior for many purposes. For example, susceptibility to some viruses can be species specific and some physiological processes can vary between species, leading to different sensitivities to stimuli. Thus, a cell line from the *A. rostrata* would be a valuable tool for studying species specific responses at the cellular level.

Over the last 25 years, a small number of cell lines with valuable properties have arisen spontaneously *in vitro* from the peripheral blood (PB) of fish. At this time it is unknown how common this phenomenon is among fish, or vertebrates in general, the nature of the cells giving rise to them, and the full potential of the cell lines to differentiate and express functional properties. The first PB cell line was derived from the common carp (Faisal and Ahne, 1990), but most PB cell lines have been from the catfish, including cell lines expressing properties of monocyte/macrophages (Vallejo et al., 1991), NK cells (Shen et al., 2002), T lymphocytes (Hogan et al., 1999) and B lymphocytes (Miller et al., 1994). A likely origin of PB cell lines is from circulating stem cells, although contamination of the blood with cells from the blood vessel wall or overlying tissue is another, albeit remote possibility. In human PB, circulating stem cells include adult pluripotent stem cells, mesenchymal stem cells, and endothelial progrenitor cells (Khakoo and Finkel, 2005; Roufosse et al., 2004; Zhao et al., 2003). However, the nature of circulating stem cells in the blood of poikliotherms is unknown and unlikely to be demonstrated directly in the foreseeable future due to the absence of identifying cell surface markers and the difficulty and cost of developing them. By contrast, demonstrating the routine development of

particular types of cell lines from fish PB would be indirect and inexpensive evidence for the presence of specific kinds of circulating stem cells, as well as providing cell lines for a variety of purposes, such as studying viruses.

For these reasons, the culturing of blood cells from the American eel (*A. rostrata*) was explored, resulting in the development and characterization of a peripheral blood leukocyte eel cell line, PBLE. This cell line demonstrated unique growth characteristics, susceptibility to Chum salmon reovirus (CSV), a member of the genus *Aquareovirus*, and the ability to die *via* apoptosis.

7.2. MATERIALS AND METHODS

7.2.1. Culture Initiation

Peripheral blood leukocytes (PBLs) were obtained from healthy American eel, *Anguilla rostrata.* The eels were anesthetized in a 100 mg/L clove oil solution (Anderson et al., 1997). Blood was obtained by venipuncture of the caudal blood vessel, transferred to a 15 mL polypropylene centrifuge tube (Falcon/Becton-Dickinson, Franklin Lakes, NJ) and put immediately on ice. The cells were centrifuged in a swinging bucket rotor centrifuge at 150-200 xg for 8 minutes at room temperature. Centrifugation resulted in the separation of blood into plasma and red blood cells with a buffy coat layer of white blood cells, which was removed using a glass pasteur pipette. These cells were put into a 50 mL polypropylene centrifuge tube (Falcon) with ~45mL collection media (Leibovitz's L-15 medium with 2% fetal bovine serum (FBS), 2% penicillin/streptomycin solution, and 10 IU heparin/mL, all Sigma, St. Louis, MO). After gentle mixing by inversion to distribute cells evenly, the cell suspension was distributed into 6 15 mL centrifuge tubes, approximately 7mL aliquots. Three mL of Histopaque® 1077 (Sigma) was layered under the 7mL cell suspension using a 9" pasteur pipette. This layered solution was centrifuged in a swinging bucket centrifuge at 400 xg for 30 minutes. The leukocytes, which form a white band at the interface of the Histopaque and L-15, were collected with a pasteur pipette, pooled, and washed in growing medium (L-15 containing 20% FBS, and 2% penicillin/streptomycin). The cells were resuspended to $2.5x10^7$ cells in 5 mL growing medium in a 12.5 cm^2 tissue culture flask (Falcon), and incubated at 21°C. Following an undisturbed year at 21°C, a colony of fibroblast-like cells was observed at the end of one of the flasks. This culture was trypsinized, split 1:2, and observed. A confluent monolayer formed 5d after passaging.

7.2.2. Routine cell culturing and maintenance

PBLE growing medium is L-15 containing 20% FBS and 2% penicillin/streptomycin. When reaching confluency, which is approximately every 7-10 d, cells are subcultured at a ratio 1:2 according to the standard trypsinization method, using 0.5mg/mL trypsin. The PBLE cell line has been cryopreserved in liquid nitrogen when suspended in growing medium with 10% dimethyl sulfoxide (DMSO; Sigma) as described previously for cell lines (Bols and Lee, 1994) and has been successfully thawed.

7.2.3. Cell growth characteristics

The optimal growing conditions for the PBLE cell line were obtained by determining attachment efficiency, as well as temperature and FBS concentration preferences.

For attachment efficiency, the number of attached cells from the number seeded (expressed as a percent) was determined at different starting cell densities and FBS concentrations. PBLE cells were plated in 12 well tissue culture plates (Falcon) at $5.0x10^4$, $1.0x10^5$, $1.5x10^5$ and $2.0x10^5$ cells per well with three replicates per cell density. The total volume of each well was brought to 1mL medium (L-15, 2% penicillin/streptomycin), one plate using growing medium with 20% FBS, one plate without FBS. Plates were incubated at 21°C for 4 hours to allow cells to attach. After which adherent cells were detached using trypsin and counted using a Coulter particle counter (Beckman, Mississauga, ON).

Optimal growing serum concentrations were determined by plating PBLE cells at a density of $5.0x10^4$ cells per well in 12 well plates with 1mL growing medium. After an incubation at 21°C for 24 hours to allow cells to attach, the growing medium was removed and 1mL new medium containing varying concentrations of FBS was added (0, 10%, 15% and 20% FBS). There were three replicates for each FBS concentration. The cells, incubated at 21°C, were collected by trypsinization at 3 day intervals and counted using a Coulter particle counter.

Temperature preference was determined using a similar experimental design. Cells were plated in normal growth medium as described above. The cells were incubated at 21°C for 24 hours to allow the cells to attach, after which the cultures were moved to different temperatures: 5°C, 12°C, 21°C, 28°C and 30 °C. Cultures were also placed at 36°C for observation, but cell counts were not quantified. Temperature experiments were performed using normal growing medium supplemented with 20% FBS. Cells were collected and counted as described above at 3 day intervals.

7.2.4. Chromosome preparations

Metaphase chromosome spreads (n=66) were prepared and counted from PBLE cultures at 112 population doublings. Standard procedures, with some variations, were used (Ganassin et al., 1999; Freshney, 1994). Briefly, cultures with dividing cells were exposed to 0.4 µg/ml demecolcine (Sigma) for 2.5 h, trypsinized and centrifuged at 400 x g for 10 minutes. The supernatant was removed and the cell pellet was suspended in 0.075 mol/L KCl (Sigma) for 8-10 minutes after which the cells were subsequently collected by centrifugation. The pellet was fixed in 3:1 methanol-glacial acetic acid for 6 h at 4 °C, collected again by centrifugation, and resuspended again in the fixative. Slides were prepared by dropping 2-3 drops of cell suspension onto a glass slide (VWR, West Chester, PA) from a 2-3 foot height, stained with 4% Giemsa stain (Sigma), and observed using a Nikon Optiphot microscope (Japan). The number of chromosomes present per cell was counted under oil immersion.

7.2.5. EROD induction

PBLE was tested for expression of P450 enzymes *via* 7-ethoxyresorufin *O*-deethylase (EROD) activity. Cells were plated in 96 well plates (Falcon) at $3.0x10^5$ cells per well and were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or benzo[a]-pyrene (BaP) for 24h before being tested for EROD activity as previously described (Bols et al., 1999). TCDD concentrations ranged from 0.8 to 3123 pmol/L, while BaP concentrations ranged from 2.8 to 23 040 pmol/L. EROD activity could not be detected with either treatment (data not shown).

7.2.6. Respiratory burst assay

PBLE were plated at $3.0x10^5$ cells per well in a 96 well plate and allowed to attach overnight. Cells were then challenged with lipopolysaccharide (LPS; 0.01-100 μg/mL; Sigma) or phorbol 12-myristate 13-acetate (PMA; 0.2 ng/mL-2 μg/mL; Sigma) in Dulbecco's phosphate buffered saline (D-PBS; Sigma) supplemented with D-glucose (500 mg/L; Sigma) and immediately measured for a respiratory burst using the fluorescent indicator dye, 2',7'-dichlorodyhydrofluorescein (H_2DCF; Molecular Probes, Eugene, OR), as previously described (Brubacher and Bols, 2001). Oxidation of H_2DCF was measured using a fluorometric plate reader (Molecular Devices, SpectraMax). Excitation and emission wavelengths were 485nm and 530 nm, respectively. Measurements were taken every 5 minutes for 4h, and then every hour for 24h. Relative fluorescent units (RFUs) from control and treated cultures were compared, and no significant differences could be detected with either treatment (data not shown).

7.2.7. CSV exposure

Chum salmon reovirus (CSV) was obtained from ATCC and routinely propagated on CHSE-214 monolayers. Media was collected 7d post-infection (pi), passed through a 0.2 μm filter, and kept frozen at -80°C until used. For all experiments, PBLE were grown to 90% confluency in $25cm^2$ tissue culture treated flasks (Nunc, Roskilde, Denmark), and the media was changed to 5% FBS immediately prior to virus infection. Cells were infected with $10^{4.3}$ tissue culture infectious dose $(TCID)_{50}$/mL, as determined using the Karber method (Karber, 1931).

7.2.8. CSV replication assay

A CSV replication assay was performed as previously described (chapter 6). Briefly, CSV was added to a confluent monolayer of PBLE ($10^{4.3}$ $TCID_{50}$/mL) for 2h to allow for viral attachment. The media was then removed, the cultures were washed twice with D-PBS and fresh growth media was added to each culture, supplemented with 5% FBS. These cultures were incubated for either 4d or 7d, after which the media was collected, passed through a 0.2μm filter, put on a fresh monolayer of CHSE-214, and incubated for 3d to measure syncytia formation.

Intracellular infectious particles were detected by lysing the cells by 3 freeze/thaw cycles (5 minutes at -80°C and 5 minutes at 37°C), followed by resuspension of the cell lysate in L-15 with 5% FBS. This cell lysate was filtered (0.2µm), put on fresh CHSE-214 monolayers and assayed for syncytia formation. Syncytia formation on CHSE-214 would suggest that PBLE supports CSV replication.

7.2.9. Cytochemical staining of syncytia

Cultures were stained to demonstrate syncytia formation using a Romanowsky-Giemsa stain. PBLE cultures exposed to CSV or CHSE-214 reporter cultures from the CSV replication assay were fixed in anhydrous methanol for 10 minutes, washed in distilled water for 1 min and allowed to air dry. The slides were then submerged in stain (3 mg/mL azure B bromide in DMSO, 1 mg/mL eosin Y in anhydrous methanol, diluted 1:15 in 10mM HEPES buffer [10mM HEPES, 5% DMSO, pH 6.8]) for 30 minutes. Azure B bromide was purchased from Manufacturing Chemists (Norwood, OH), and eosin Y was purchased from Allied Chemical (Morriston, NJ). Slides were then rinsed in distilled water for 1 minute, and allowed to air dry. Slides were mounted using a non-aqueous mounting medium and visualized using a Nikon Optiphot microscope.

7.2.10. Cell viability

Two fluorescent indicator dyes, alamar Blue™ (Medicorp, Montreal, PQ) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Molecular Probes) were used to evaluate cell viability. The alamar Blue dye measures cellular metabolism (resorufin) (O'Brien et al., 2000), while CFDA-AM measures intracellular esterase activity, thus confirming membrane integrity (Schirmer et al., 1997). The levels of fluorescence are measured using a microplate spectrofluorometer. Alamar Blue and CFDA-AM assays were performed as previously described (Ganassin et al., 2000). Briefly, cells were plated at $3.0x10^5$ cells per well in a 96 well plate and allowed to attach overnight. Cells were then exposed to serial dilutions of CSV (initial titre=$10^{4.3}$ $TCID_{50}$/mL) or gliotoxin (5µg/mL to 0.5 ng/mL) for a specified time period prior to measuring cell viability. After this exposure, the media was removed and the two fluorescent dyes were added to the wells, diluted in a minimal salt solution called L-15ex. The cells were incubated with the dyes for 60 minutes before the microwells were read with a fluorometric plate reader (Molecular Devices, SpectraMax). Excitation and emission wavelengths were 530 and 595 nm for alamar Blue and 485 and 530 nm for CFDA-AM. Cytotoxicity was indicated by a decline in fluorescence units (FUs) for treated cultures relative to untreated control cultures. FUs for culture

wells with no cells were constant and subtracted from FUs for experimental and control cultures. For graphical presentation, the results were plotted using SigmaPlot (Jandel Scientific).

7.2.11. gDNA laddering assay

Cells plated to confluency in 25cm^2 tissue culture treated flasks and allowed to attach over night. Cells were then exposed to either $10^{4.3}$ $TCID_{50}$/mL CSV with or without 50 μg/mL ribavirin or increasing concentrations of gliotoxin, for a specified length of time. After the specified treatment, cells were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit (Sigma), which is based on column affinity technology. The genomic DNA was eluted from the column using 100 μL Milli-Q water, and 35 μL of this DNA was resolved by electrophoresis on a 2% (w/v) agarose gel for 4 hours at 60V. The DNA ladders were visualized by staining gels with 0.5 μg/mL ethidium bromide and photographed under a UV transillumination.

7.3. RESULTS

7.3.1. Culture morphology

PBLE was derived from a peripheral blood leukocyte preparation using a technique to preferentially isolate the monocyte/macrophage population. Out of this culture arose a continuous cell line of fibroblast morphology (Figure 7-1A), which appears to be homogenous in cell type. Older cultures produce phase bright foci or 'centres' (Figure 7-1B and C), that generate phase bright round cells. These phase bright cells remain in suspension but are able to reattach in a new culture flask (Figure 7-1D).

7.3.2. Culture growth

The growth of cultures required that the L-15 medium be supplemented with fetal bovine serum (FBS). Both cell attachment and proliferation was influenced by FBS. At lower cell densities, cells attached similarly, with or without FBS; however, at higher cell densities FBS aided in cell attachment (Figure 7-2). With time, cell number increased slightly or not at all in cultures with L-15 alone (Figure 7-3). PBLE proliferated better with more FBS as tested to 20% FBS (Figure 7-3).

Temperature also influenced the growth of cultures. PBLE grew best at 21 and 28^{o}C. At 30 °C a slight increase in cell number occurred, and although cell number was not measured at 36 °C, cells remained attached to the culture wells for at least 7 days. Cells maintained at 5^{o}C and 12^{o}C persisted but did not proliferate (Figure 7-3 B). It was concluded that culture grew best with 15-20% FBS supplementation (Figure 7-3A) and at 21-28^{o}C (Figure 7-3B).

Culture morphology changed at different maintenance temperatures (Figure 7-4). After a week at the temperature extremes, 5 and 36°C, monolayers of PBLE remained intact, although monolayer appearance changed subtly. With time at 5 °C, the cells became shorter and rounded, whereas at 30 and 36^{o}C the cells became more epithelial-like (Figure 7-4).

7.3.3. Chromosome counts

Chromosome counts were performed after 112 population doublings. The counts ranged from 28 to 47, with a modal chromosome number of 38 (Figure 7-5), which is the normal diploid chromosome number for the American eel (Sola et al., 1980).

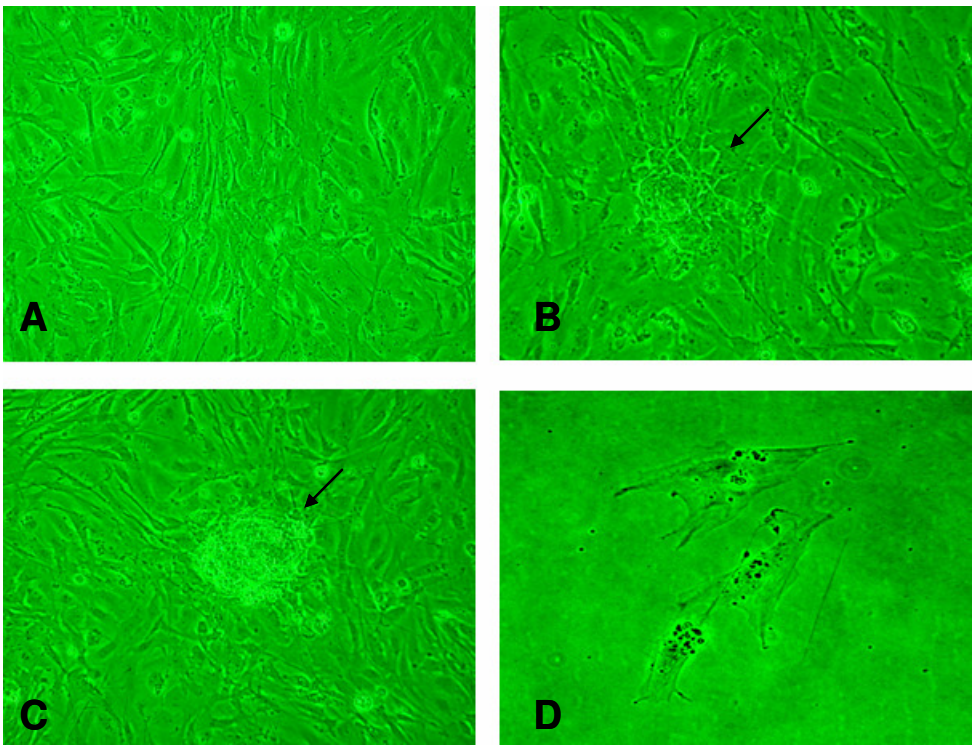

Figure 7-1. PBLE morphology in culture.

(**A**) PBLE form a monolayer of fibroblast-like cells when grown at 21^{o}C. Over time areas in the monolayer begin to grow and form 'centres' in which the cells are more densely packed (**B**). These 'centres' develop into a phase bright mound of cells (**C**), that produce phase bright viable cells that can reattach in a new culture flask (**D**). Arrows indicate forming (**B**) and more mature (**C**) 'centres'. Magnification (**A** and **C**) 100X , (**B**) 150X., (**D**) 200X.

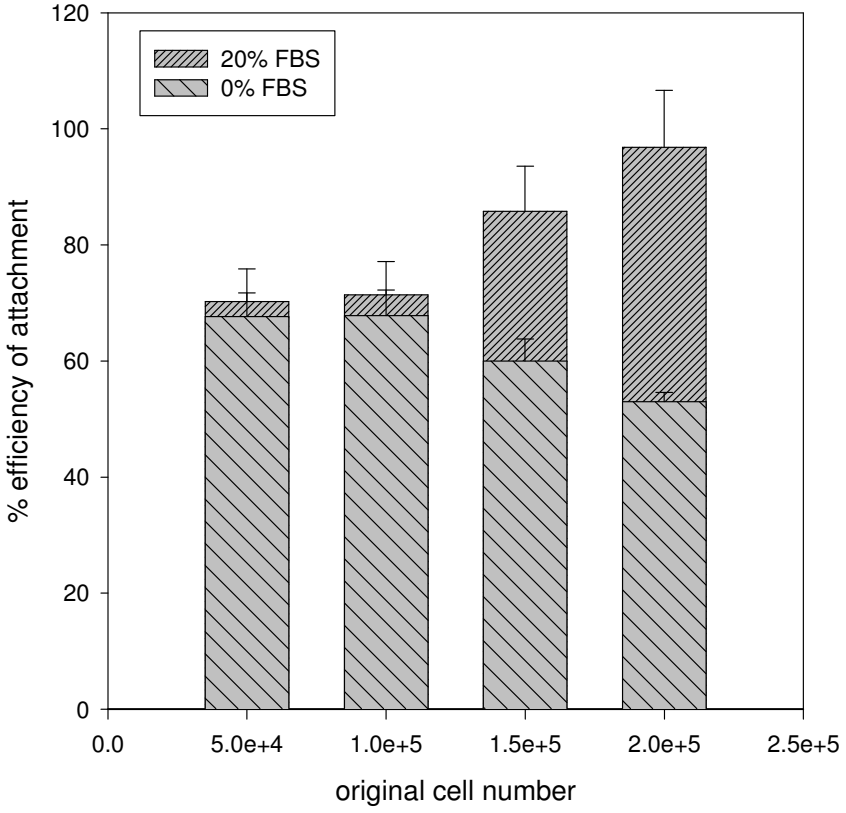

Figure 7-2. Attachment efficiency of PBLE at different cell densities.

PBLE were plated at increasing cell densities in normal growth medium with or without FBS supplementation. The number of cells able to attach 4h after plating was measured using a Coulter particle counter. Results are reported as % efficiency of attachment, a percentage of the number of attached cells compared to total number plated.

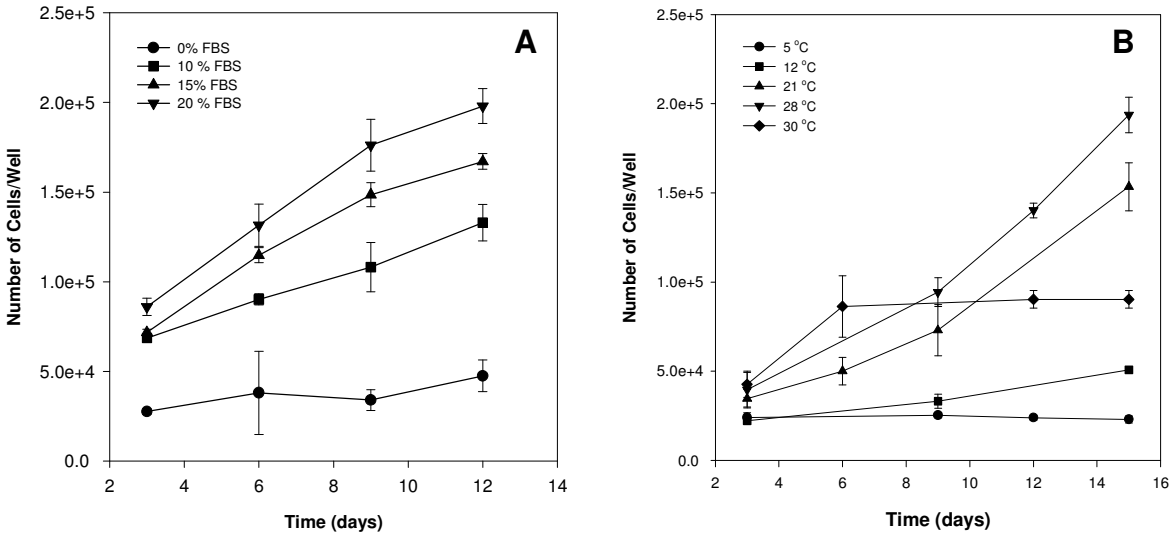

Figure 7-3. Optimal growth conditions for PBLE.

PBLE growth was measured over time in conditions of varying FBS concentration (**A**) or varying temperature (**B**). Cells were seeded at a starting cell density of $5.0x10^4$ cells per well and cell numbers were measured at specific time points using a Coulter particle counter. When grown at varying FBS concentrations, the temperature was kept constant at 21°C. When testing optimal temperature conditions, the FBS concentration was kept at 20%.

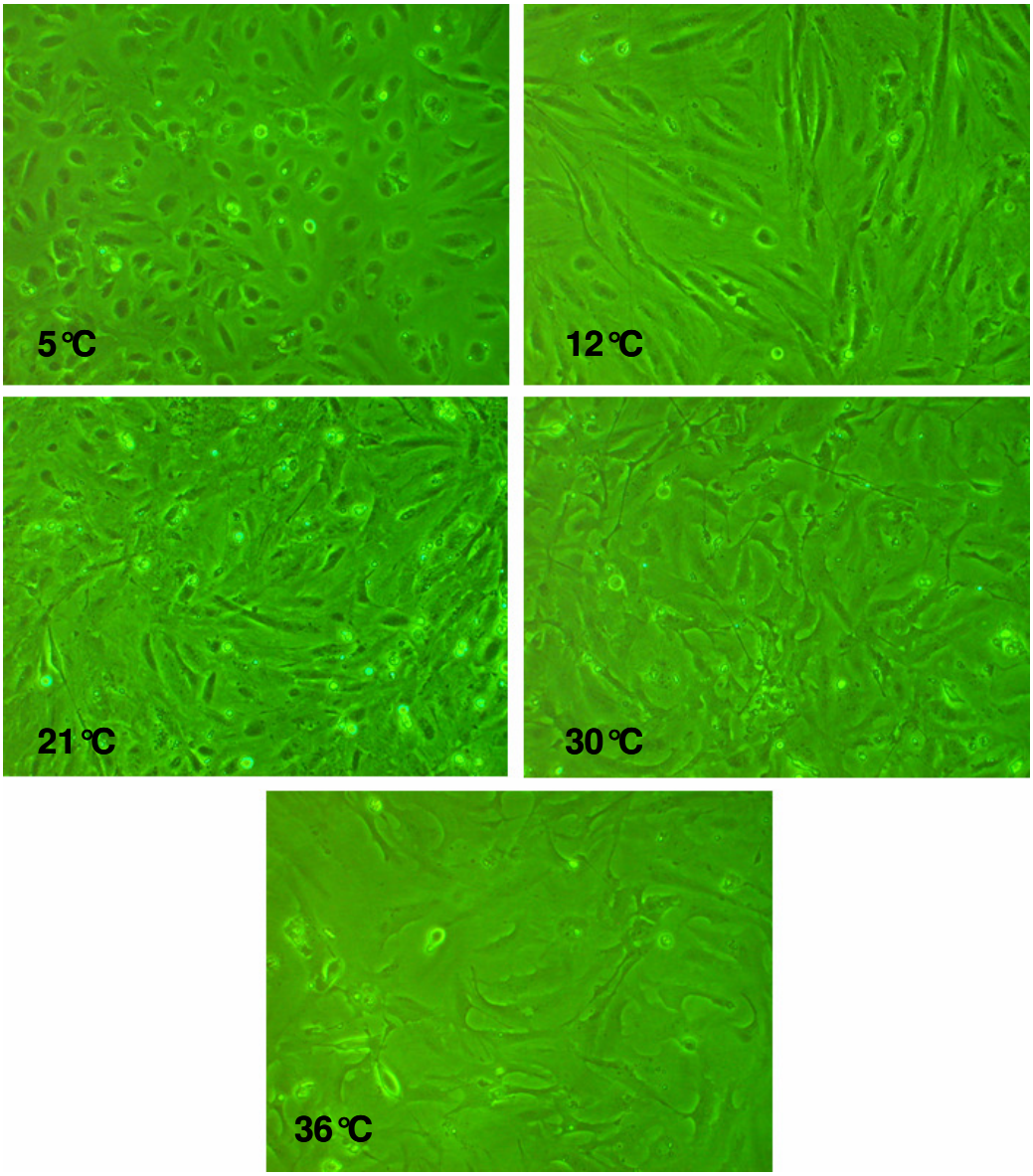

Figure 7-4. PBLE cellular morphology at different growing temperatures.

PBLE, normally grown at 21 °C and demonstrate a fibroblast morphology, changed cell shape at different temperatures. At 5°C PBLE were round, compared to 12°C where they were fibroblast-like but sparse. At 30 and 36 °C the cells flattened to a more epithelial-like morphology. All pictures taken at 3d using 150X magnification.

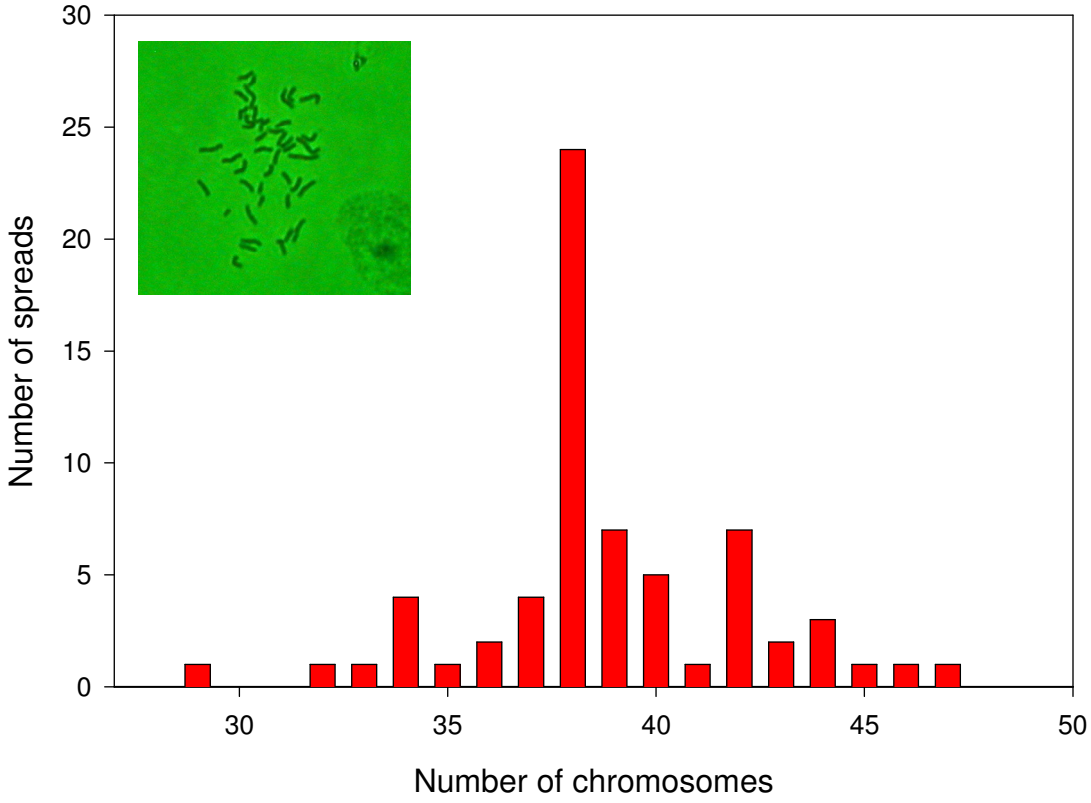

Figure 7-5. Frequency distribution of PBLE chromosomes.

It was found that after 112 population doublings, PBLE had a modal chromosome number of 38 (n=66 cells). Inset picture representative of a chromosome spread (magnification 2000X).

7.3.4. EROD induction

PBLE appeared to be negative for cytochrome P-450 enzymes as measured by 7-ethoxyresorufin-O-deethylase (EROD) activity in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or benzo[a]-pyrene (BaP) (data not shown).

7.3.5. Respiratory burst

PBLE did not undergo a respiratory burst in response to lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA), as measured using the fluorescent indicator dye, 2',7'-dichlorodihydrofluorescein (H_2DCF) (data not shown).

7.3.6. Chum salmon reovirus (CSV) susceptibility

PBLE were susceptible to CSV and demonstrated CPE following viral exposure. CSV exposed PBLE began to fuse and form syncytia within 3 d pi, and showed mature syncytia formation by 5 d pi (Figure 7-6). Late syncytia do not lift off the growing surface, as is observed with other fish cell lines, but remain adherent with an intact cell membrane (data not shown). A replication assay demonstrated the ability of PBLE to support CSV replication (Figure 7-7). By 4d pi viral particles could be detected within the cells but very few in the media, while by 7d pi a higher tire of infectious virions could be detected in both the media and within the cells. CSV-induced syncytia formation did result in cell death (Figure 7-8 A); however, this cell death did not appear to be *via* apoptosis as determined by gDNA laddering (Figure 7-8B).

7.3.7. Gliotoxin-induced cell death via apoptosis

PBLE died following a 24h exposure to gliotoxin, a fungal secondary metabolite, as measured using fluorescent indicator dyes, alamar Blue and CFDA-AM (Figure 7-9A). This gliotoxin-induced cell death was *via* apoptosis as determined by gDNA laddering into 180bp oligomers, a hallmark of apoptosis (Figure 7-9B) (Mesner and Kaufman, 1997).

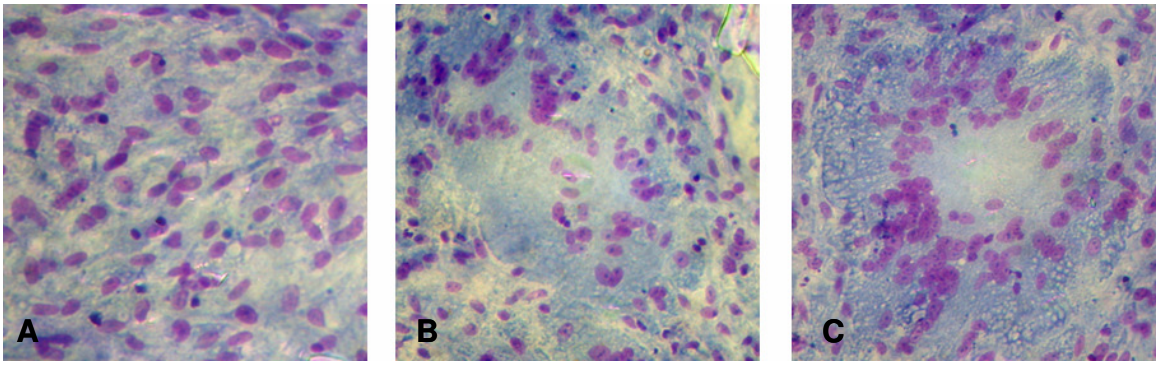

Figure 7-6. Chum salmon reovirus (CSV) induced cytopathic effect (CPE) in PBLE.

The CSV induced CPE in PBLE, syncytia formation, became evident by 3d pi (**B**) and developed into mature syncytia by 5d pi (**C**). Control cultures show a healthy monolayer of cells (**A**). All pictures taken at 200X magnification.

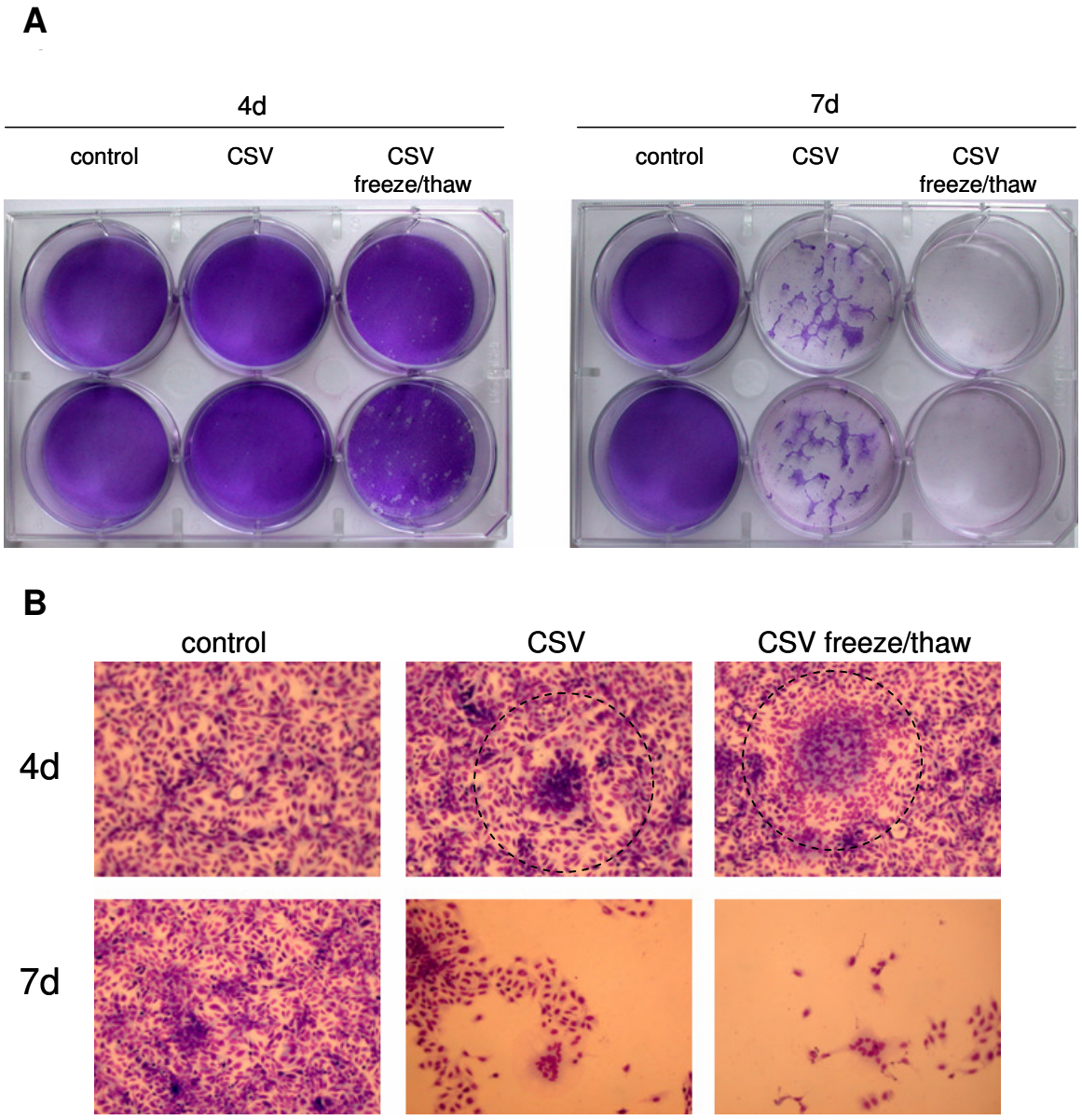

Figure 7-7. CSV replication in PBLE.

PBLE were exposed to CSV ($10^{4.3}$ $TCID_{50}$/mL) for 2h, after which the virus containing media was removed and fresh media was added. The cells were incubated for either 4 or 7d pi. The conditioned media or cell lysate (derived from freeze/thaw cycles) was added to new CHSE-214 monolayers and allowed to incubate for 3d before syncytia formation was assessed by Romanowsky stain. **A** Whole plate pictures of CHSE-214 reporter cultures show the differences between 4d and 7d pi and between virions detected extracellularly (CSV) and intracellularly (CSV freeze/thaw) from infected PBLE cultures. **B** Romanowsky-Geimsa stained CHSE-214 show syncytia formation at 4d pi (syncytia outlined with dashed circle) and the breakdown of large syncytia by 7d pi (100X magnification).

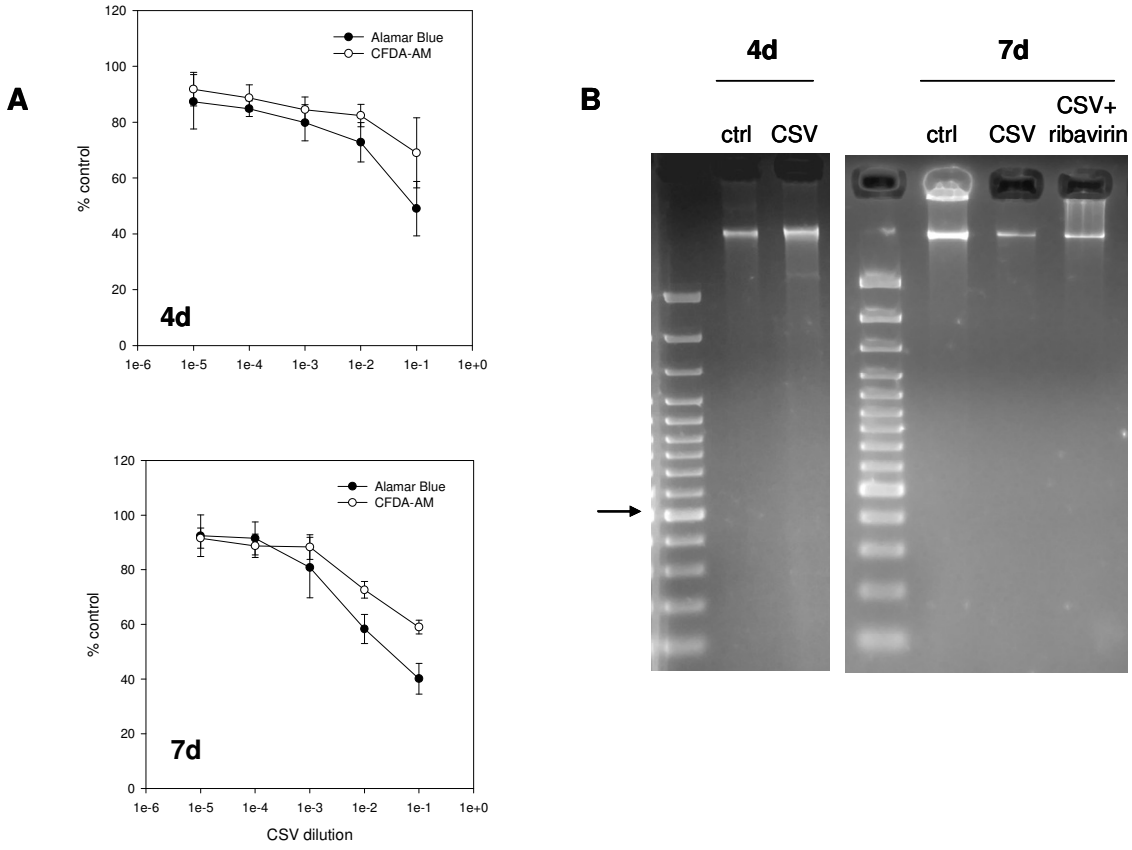

Figure 7-8. CSV induced cell death in PBLE by a mechanism other than apoptosis.

Cell viability was assessed in PBLE cultures exposed to serial dilutions of CSV (initial titre=$10^{4.3}$ $TCID_{50}$/mL) for 4 or 7d using two fluorescent indicator dyes (**A**). Genomic (g)DNA from control and virus exposed cultures with or without 50µg/mL ribavirin, an inhibitor of viral transcription, was run on a 2% agarose gel. gDNA laddering in 180bp oligomers, a hallmark of apoptosis, could not be detected (**B**). An arrow points to the 500 bp band in the lane containing the 100bp DNA marker.

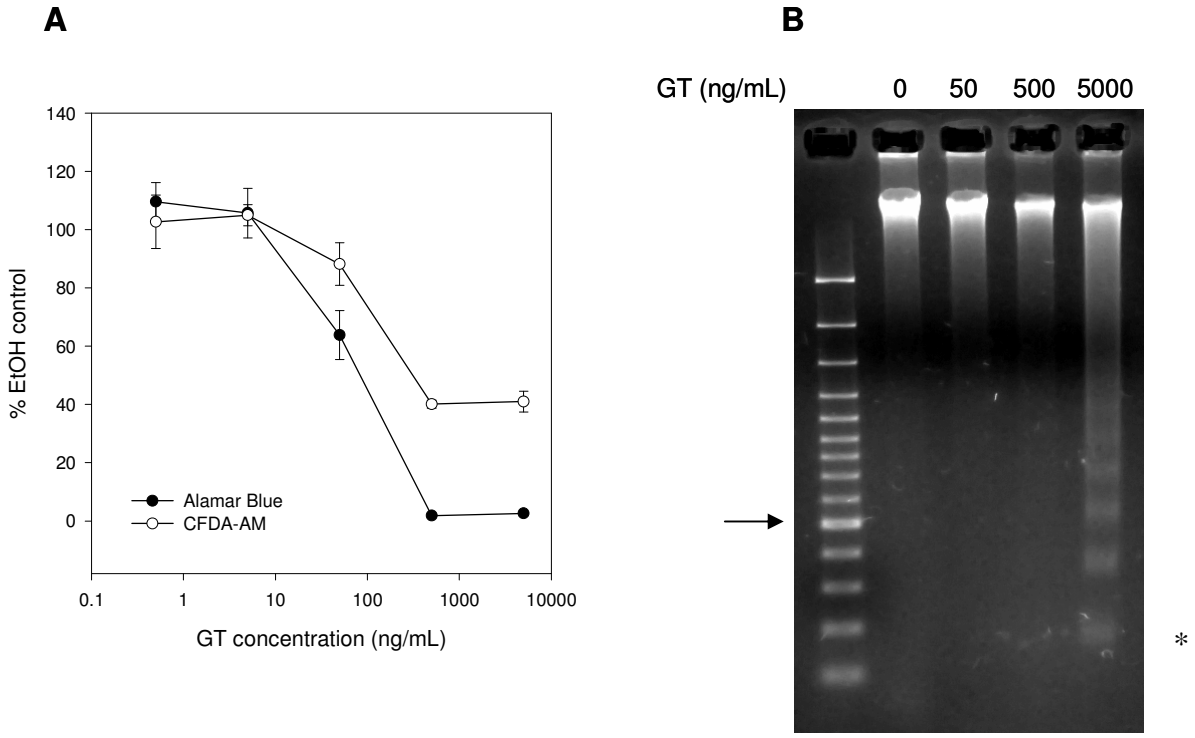

Figure 7-9. Effect of gliotoxin, a fungal secondary metabolite, on PBLE cell viability.

Cell viability was measured in PBLE cultures exposed to gliotoxin after 24h exposures (**A**). gDNA laddering, a hallmark of apoptosis, was measured from PBLE cultures treated with increasing concentrations of gliotoxin (GT) for 24h (**B**). An asterisk identifies the position of the 180 bp fragment and an arrow points to the 500 bp band in the lane containing the 100bp DNA marker.

7.4. DISCUSSION

PBLE is the first continuous cell line developed from *A. rostrata*, and arose from a peripheral blood leukocyte preparation. Cell lines have been previously derived from the adherent cells of catfish and carp peripheral blood; however, PBLE differs from them in several ways. Channel catfish peripheral blood leukocytes have produced several monocyte-like cell lines (Vallejo et al., 1991). These cell lines had the characteristic morphology of monocytes/macrophages and were capable of several monocyte/macrophage functions, including phagocytosis, interleukin-1 secretion and antigen presentation (Vallejo et al., 1991). CLC is a cell line developed from the mononuclear cells of carp peripheral blood (Faisal and Ahne, 1990). Even though these cells did not have a typical monocyte/macrophage morphology they did react to a monoclonal antibody raised against carp macrophages and expressed at least two macrophage functions: phorbol myristate acetate (PMA) and lipopolysaccharide (LPS) caused a respiratory burst and CLC secreted interleukin-1 (Faisal and Ahne, 1990; Weyts et al., 1997). By contrast, PBLE did not show typical monocyte/macrophage morphology nor were they capable of a respiratory burst. Under routine culture conditions PBLE had a bipolar or fibroblast-like morphology

PBLE might be derived from fish equivalents of several types of circulating cells that have been identified in mammalian blood and that grow *in vitro* as adherent cells with morphologies different from typical monocytes. Among these are circulating endothelial progenitor cells (EPCs), as well as mature circulating endothelial cells (CECs) (Khakoo and Finkel, 2005). Smooth muscle progenitor cells (SPCs) also have been found in human peripheral blood (Simper et al., 2002). Pluripotent stem cells have been grown *in vitro* from human and mouse leukocytes, particularly from different subsets of monocytes. These include f-macrophages, which could be induced to differentiate into five distinct cell lineages (Zhao et al., 2003), and mesenchymal stem cells or progenitor cells (MPCs) capable of expressing properties of osteoblasts, skeletal myoblasts, chondrocytes and adipocytes (Kuwana et al., 2003; Roufosse et al., 2004). The relationship between these stem cell types is still incompletely understood. All of them can be expanded *in vitro* and in some cases cell lines have been developed (Conrad et al., 2002).

The absence of antibodies to cell surface antigens and to functional proteins of differentiated cells makes identifying the origin of PBLE and their differentiation potential difficult to determine at this time. However, some general properties can be considered. PBLE were found to be negative for EROD activity. A previous study showed differential EROD activity in various eel cell subtypes suggesting that EROD activity can be detected in eel cells (Garrick et al., 2005).

Also, PBLE share some characteristics with MPCs. PBLE could be removed from the growth surface by conventional trypsin treatment, which was true for MPCs but not for f-macrophages (Zhao et al., 2003). Like the mesenchymal stem cell line, V54/2, (Conrad et al., 2002), PBLE cultures produced a small number of viable, round floating cells, which were able to reattach and proliferate in a new culture flask.

Peripheral blood cells of fish definitely appear to be a potential source of cell lines but how easily and universally they can be generated is still uncertain. From the success so far, the blood of fish can be assumed to contain some circulating stem or progenitor cells. Thus, one contributing factor to success would be the number and type of circulating stem cells, which might vary with the health status of the fish. In humans for example, CECs are elevated in patents with septicemia (Mutunga et al., 2001), and MPCs can be mobilized by treatment with granulocyte-monocyte colony stimulating factor (GM-CSF) (Roufosse et al., 2004). Another factor for cell line development would be how easily the cells spontaneously immortalize once in culture. In the case of the catfish (Vallejo et al., 1991), the success rate appeared high, whereas with the carp and eel, cell line development appeared to be a rare event. In the case of human and rodent peripheral blood cells, direct immortalization has been carried out with SV40 large-T-antigen leading to mesenchymal stem cell lines (Conrad et al., 2002). Application of such direct immortalization procedures to fish peripheral blood cells might lead to cell lines being obtained more frequently.

Regardless of their cellular origin or differentiation potential, PBLE offer for the first time a cell line from *A. rostrata* and a tool for studying viral-host interactions specific to the American eel. Upon exposure to Chum salmon reovirus (CSV), PBLE illustrated the cytopathic effect (CPE) that is often characteristic of aquareoviruses, which is the formation of syncytia (Lupiani et al., 1995). Such a clear microscopic endpoint could make the cell line useful for evaluating tissue extracts for viral presence. PBLE were also able to support CSV replication, making this cell line valuable for studying viral replication mechanisms as well as CPE. Viral exposures also lead to cell death in PBLE cultures after 4d exposures to CSV.

Apoptosis is a common form of cell death but is not a capability of all cell lines (Kolenko et al., 1999; DeWitte-Orr and Bols, 2005). PBLE was able to undergo apoptosis as determined by gliotoxin-induced gDNA laddering. Gliotoxin is a fungal metabolite and cytotoxic agent that usually kills by apoptosis and is often most potent to monocyte/macrophages (Stanzani et al., 2005; DeWitte-Orr and Bols, 2005). Apoptosis is a frequent form of reovirus-induced cell death (Clarke and Tyler, 2003). However, the death of PBLE in response to CSV did not appear to be by apoptosis as no gDNA laddering was detected. Further experiments need to be performed to

determine the precise mechanism of CSV-induced death of PBLE, but the results do imply that apoptosis is not absolutely necessary for viral replication and release from this cell line.

The PBLE cell line appears to be unique among fish cell lines in surviving 5 to 36 °C and proliferating from room temperature to 30 °C, which are temperatures that encompass the ranges for cell lines of both coldwater and warmwater fish. Cell lines from coldwater fish proliferate at temperatures as low as 5 °C and as high as up to 24- 26 °C (Fryer and Lannan, 1994; Bols et al., 1992). Cell lines of warmwater fish generally grow optimally around 30 °C (Chen et al., 1988). The American eel is found from 5 ° to 62° 18'N latitude (Scott and Crossman, 1973), which is a geographical range that would contain habitats with a wide range of temperatures. The temperature tolerance of the American eel could be exceptional and PBLE could be useful in exploring cellular and biochemical mechanisms responsible for this. Also, as pointed out by several authors (Nicholson et al., 1987; Kang et al., 2003), cell lines that grow over a wide range of temperatures are potentially valuable for isolating and studying both coldwater and warmwater fish viruses. Thus, PBLE may prove to be a valuable tool for understanding many eel specific characteristics of temperature tolerance, cell death pathways and antiviral mechanisms.

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Chapter 8

Conclusions and Future Aims

CONCLUSIONS AND FUTURE AIMS

It is clear that viral infections have devastating effects on wild and cultured fish populations. It is also clear that an effective control measure to prevent infection needs to be developed. Current research has focused on identifying and characterizing type I interferon and subsequent interferon inducible genes in fish, to better understand this potent antiviral pathway. Though in its early stages, this research has found that fish type I interferon (IFN) functions similarly to mammalian IFN, though structurally dissimilar. Also, the ISGs that facilitate the intracellular antiviral state have begun to be isolated, and also seem to function similarly to what is known in mammals. The complete characterization of this pathway will facilitate the creation of better preventative measures to protect fish stocks from pathogenic viral diseases.

In this study, antiviral mechanisms were assessed using the rainbow trout macrophage-like cell line, RTS11, and compared with other teleost cell lines. Apoptosis was compared as an antiviral *versus* an anti-fungal response. Altered gene expression of *Mx* and *vig-1*, as well as HA induction, was assessed following dsRNA and viral infections. Cellular responses to the Chum salmon reovirus (CSV) were studied and compared between three teleost cell lines, RTS11, RTG-2 (rainbow trout gonadal) and CHSE-214. Finally, antiviral mechanisms were investigated in a newly developed cell line from American eel (*Anguilla rostrata*), PBLE. Many new avenues of study can be explored based on what has been found thus far, a few of which are described below.

8.1. A COMPARISON OF dsRNA *vs.* GLIOTOXIN-INDUCED APOPTOSIS

8.1.1. Macrophage sensitivity

In this study, apoptosis was studied in RTS11, RTG-2 and CHSE-214 following exposures to dsRNA (poly IC) alone or in combination with macromolecular inhibitors and to gliotoxin. The evidence suggests that RTS11 was more sensitive to the pro-apoptotic stimuli and died by apoptosis at lower concentrations of stimuli and more thoroughly within a culture when compared to RTG-2 and CHSE-214. It is possible that this data suggests that salmonid macrophages are more predisposed to apoptosis than fibroblast or epithelial cell types. This is an attractive idea, that immune cells, being at the front line of defense, are more susceptible to cell suicide if infected by a pathogen. This would ensure that infected immune cells would not be able to circulate throughout the body, spreading the infection. In effect, it would be similar to spies being forced to take a suicide pill upon capture. Another interpretation is that this sensitivity could reflect the role of apoptosis as a control mechanism, killing activated immune cells to prevent a prolonged inflammatory response (Malyshev and Shnyra, 2003). Conversely, it could be said that the stimuli chosen catered more to macrophage specific apoptosis, as macromolecular inhibitors

selectively killed mammalian macrophages (Lewis et al., 1995), the suggested cFLIP pathway could also be a macrophage-specific response (Perlman et al., 1999) and gliotoxin has been reported as being a macrophage specific inducer of apoptosis (Schwartzman and Cidlowski, 1993). More research is necessary using cell lines and primary cultures of immune and non-immune cells to determine whether the suggested macrophage sensitivity to apoptosis is the case in fish.

8.1.2. Two pathways to death

It is possible that poly IC and gliotoxin induced apoptosis in RTS11 *via* two separate pathways; the extrinsic, or death receptor mediated pathway, and the intrinsic, or mitochondrial mediated pathway, respectively. As described in this thesis, poly IC-induced cell death seemed to involve PKR. In mammals, dsRNA-activated PKR appears to induce apoptosis using the death receptor-mediated pathway. PKR-mediated apoptosis is associated with increased levels of Fas and FADD, as well as caspase 3 and 8. Interestingly, PKR-mediated DISC formation did not require FasL production and release (Gil and Esteban, 2000b; Balachandran et al., 1998). Although the mitochondria may be involved in dsRNA induced apoptosis, caspase 9 and the mitochondrial apoptotic pathway seem to lie downstream of caspase 8 and the death receptor pathway in PKR induced apoptosis (Gil et al., 2002). On the other hand, gliotoxin is associated with the mitochondria-mediated pathway. Gliotoxin induces DNA damage by reactive oxygen species production (ROS) (Eichner et al., 1988); a classic inducer of the intrinsic pathway. Furthermore, gliotoxin-induced cell death is associated with mitochondrial membrane destabilization and cytochrome c release in human hepatic stellate cells (Kweon et al., 2003) and in murine macrophages (Suen et al., 2001).

Preliminary data using specific inhibitors of caspase 8 and 9 in combination with poly IC and cycloheximide or gliotoxin suggest similar pathways are being activated in RTS11.

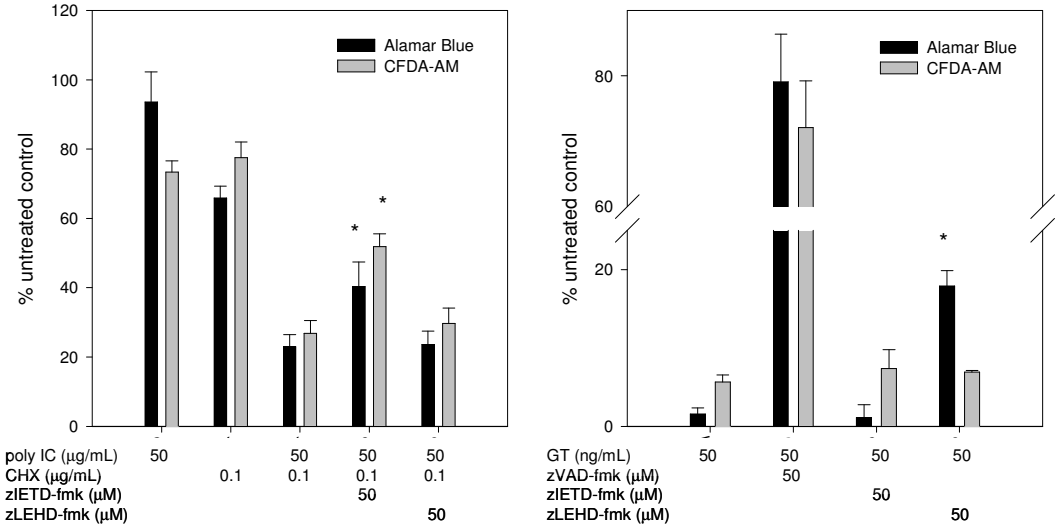

Figure 8-1. Effect of caspase-3, -8 and -9 inhibitors in poly IC- and gliotoxin-induced cell death.

RTS11 were exposed to caspase -3 (zVAD-fmk), -8 (zIETD-fmk) and -9 (zLEHD-fmk) inhibitors 30 minutes prior to exposures with either 50 µg/mL poly IC and 0.1 µg/mL cycloheximide (CHX) or 50 ng/mL gliotoxin (GT). Cell viability was measured using two fluorescent indicator dyes, alamar Blue and CFDA-AM. Inhibitor treatments significantly different from the control (poly IC+CHX, or GT alone) were determined using a one-way ANOVA with a Tukey post test (* $p<0.001$).

The caspase 8 inhibitor partially inhibited cell death by poly IC and cycloheximide treatments but not by gliotoxin as indicated by both dyes (alamar Blue and CFDA-AM), while at least with alamar Blue, the caspase 9 inhibitor blocked cell death by gliotoxin and not poly IC and cycloheximide treatments (Figure 8-1). The pan-caspase inhibitor zVAD-fmk more completely blocked both poly IC and cycloheximide (chapter 2) and gliotoxin induced cell death. This preliminary data suggests that both apoptotic pathways are present in fish, and are intact and functioning in RTS11, making this cell line a valuable tool for studying apoptosis *in vitro*.

8.2. ANTIVIRAL GENE EXPRESSION

8.2.1. Alternative roles for Mx

This study has shown that Mx expression, at both the transcript and protein level, is constitutively expressed in both RTS11 and RTG-2, albeit at different levels. These results suggest an alternative role for Mx, other than as an antiviral protein. Suggestions of non-antiviral roles for Mx occurred early on when it was discovered that Mx proteins were expressed in species not naturally infected with influenza virus, and that some Mx proteins being cloned and characterized did not demonstrate any antiviral activity (Arnheiter and Meier, 1990). One study

suggested that human Mx proteins are able to bind cellular proteins and share several properties with stress proteins (Horisberger, 1992), while another suggested that Mx proteins are essential for cellular metabolism (Arnheiter and Meier, 1990). A recent study showed that human MxA associates with a calcium-permeable cation channel and may serve as a regulatory protein in calcium signaling (Lussier et al., 2005). In fish, Mx proteins have been studied first as a marker for interferon production, and later for their antiviral activity; however, alternative roles for constitutively expressed Mx proteins have not been explored and may prove important for understanding basic cellular functions.

8.2.2. Direct *vs.* indirect pathways

Evidence in this thesis suggests that antiviral gene expression can be induced by dsRNA *via* direct and indirect mechanisms. This is the first evidence for a direct mechanism of ISG induction by dsRNA in fish, and requires further elucidation. One idea would be to pursue the role of NF-κB and IRF-3 in dsRNA induced ISG expression. IRF-3 and NF-κB are two major transcription factors activated during viral infections (Elco et al., 2005). IRF-3 is able to induce ISG expression without prior interferon induction, in other words, through the direct pathway (Sen and Sarkar, 2005). Using NF-κB inhibitors, it would be possible to block dsRNA-induced interferon production and test for interferon-independent mechanisms, such as ISG production (personal communications, Dr. K Mossman). Thus, *Mx* and *vig-1* transcript induction following co-treatments with poly IC and an NF-κB inhibitor could be further evidence for the direct pathway and suggest a role for IRF-3 in this pathway.

8.2.3. Effect of ssRNA

During the course of this study, various stimuli were used to study *Mx* and *vig-1* induction. One stimuli used was synthetic single stranded (ss) RNA, which was initially thought to be an ideal negative control for the synthetic dsRNA molecule, poly IC. However, it was found that *Mx* and *vig-1* transcripts were induced by the ssRNA molecule, poly I, at similar concentrations to those used with poly IC (Figure 8-2).

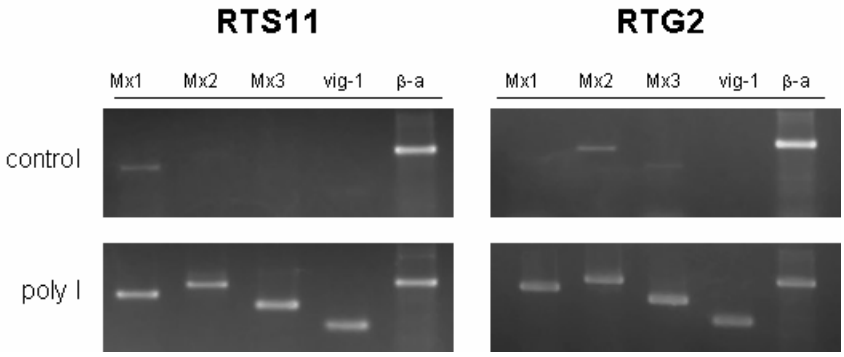

Figure 8-2. Effect of ssRNA on Mx and vig-1 transcript induction in RTS11 and RTG-2.

RTS11 and RTG-2 were treated with a synthetic ssRNA, poly I (50 µg/mL), for 24h, after which RT-PCR was performed to determine *Mx* and *vig-1* transcript levels (as described in chapter 4).

Homotypic aggregation (HA) in RTS11 was also slightly induced by a ssRNA (chapter 5). In mammals, ssRNA has been shown to induce type I interferon expression by signalling through toll-like receptor (TLR) 7 (Diebold et al., 2004; Heil et al., 2004). Taken together, HA and ISG induction by ssRNA are preliminary evidence for a similar pathway in teleost fish, which could be pursued.

8.3. HOMOTYPIC AGGREGATION

8.3.1. Involvement of galectins

In the course of the homotypic aggregation study (chapter 5) evidence suggested the involvement of galectins, a family of non-integrin adhesion molecules. An extension of this suggestion would be to monitor galectin transcript levels following poly IC exposures in RTS11 using RT-PCR. There are three members of the galectin family, the proto-type, the chimera-type and the tandem repeat-type (Rubinstein et al., 2004). All three members have been isolated from zebrafish (Vasta et al., 2004); however, only one galectin had been cloned from rainbow trout and is a galectin-9-like tandem repeat-type, molecule (Inagawa et al., 2001). It is this galectin-9-like molecule that was shown to be induced by VHSV in rainbow trout leukocytes (O'Farrell et al., 2002). Thus, primer sequences could first be developed using the known rainbow trout galectin-9 sequence; however, rainbow trout may also produce a proto-type galectin (Inagawa et al., 2001) and thus known teleost sequences from the proto-type galectins could be aligned to develop proto-type specific primers as well.

Producing recombinant rainbow trout galectins could also prove valuable. In addition to being associated with adhesion, extracellular galectins have been shown to induce apoptosis in immune cells (Rubinstein et al., 2004). It is possible that recombinant rainbow trout galectins

could induce apoptosis in RTS11 and may be an alternative inducer useful for studying apoptotic mechanisms.

8.4. CHUM SALMON REOVIRUS

When studying CSV replication in the three salmonid cell lines, RTG-2, RTS11 and CHSE-214, evidence suggested that CSV release was following syncytia formation in RTG-2 and CHSE-214, while budding without syncytia formation may have been the mechanism of viral release in RTS11 (chapter 6). A time course experiment in CHSE-214 demonstrates that extracellular infectious particles could not be detected until after syncytia formation (Figure 8-3). Infectious particles could be detected in the intracellular space by 2d pi, but extracellular virions were not present until 3d, after syncytia formation had occurred. It is interesting to note that zVAD-fmk again did not affect intracellular virion numbers, but did reduce extracellular infectious particle numbers. Thus, budding does not seem to be contributing to viral release in CHSE-214. It is the link between syncytia formation, apoptosis and virus release that could have important pathological significance and should be pursued further. One idea would be to test membrane integrity throughout syncytia formation by measuring [^{3}H] uridine leakage over time, especially in combination with zVAD-fmk, and see if membrane leakage corresponds with the presence of infectious particles in the extracellular space.

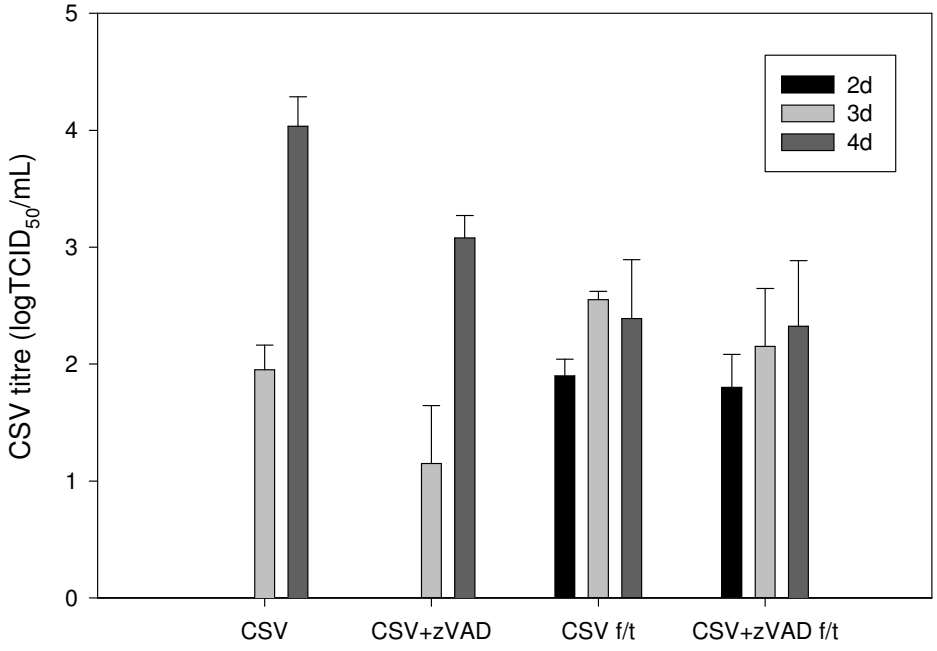

Figure 8-3. A time course of CSV replication as measured in CHSE-214.

CHSE-214 cultures were treated with CSV ($10^{4.3}$ $TCID_{50}$/mL) for 2h, after which the media was removed, fresh media with or without 50μM zVAD-fmk was added and cells were incubated for specified lengths of time. Extracellular virions (CSV, CSV+zVAD) were measured by serially diluting the media onto new CHSE-214 monolayers, while intracellular virions (CSV f/t, CSV+zVAD f/t) were detected from serial dilutions of cell lysate (lysing performed by three freeze/thaw (f/t) cycles). $TCID_{50}$ values were calculated after 4d exposures to the media or cell lysate.

Identifying the aquareovirus FAST (fusion associated small transmembrane) protein would also aid in studying CSV release. FAST-induced syncytia formation would allow for studying syncytia induced changes in membrane integrity as well as apoptosis induction without the involvement of host antiviral mechanisms and viral protein interactions.

8.5. PBLE

8.5.1. Viral susceptibility

PBLE was found in this study to be susceptible to CSV infection and to support viral replication. Previous work has shown that PBLE is not only susceptible to CSV but also other aquareoviruses (Halibut aquareovirus (AqRV) type I), rhabdoviruses (VHSV), birnaviruses (IPNV), and orthomyxiviruses (ISAV; Table 8-1).

Table 8-1. A comparison of viral susceptibility between cell lines as determined by demonstration of cytopathic effect (CPE).

Cell line	VHSV	IPNV	CSV	AqRV type I	ISAV
PBLE	+	+	+	+	+
CHSE-214	n.d.	+	+	+	-
RTG-2	+	+	+	n.d.	n.d.
RTS11	-	-	+	-	-

n.d.= no data available
Adapted from (Bryson, 2005)

This data demonstrates the versatility of PBLE in regards to viral susceptibility and shows that this cell line may prove to be a valuable tool not only for studying antiviral mechanisms but also for screening purposes. Also, *Anguilla* sp. are specifically susceptible to birnaviruses (Lee et al., 2001; Schwanz-Pfitzner et al., 1984), thus making PBLE a valuable for studying viral replication and infection of an important pathogen.

8.6. A ROLE FOR PKR

From this study it seems that PKR has a role in antiviral gene expression, homotypic aggregation and apoptosis in RTS11. This suggestion is based on inhibitor studies and measuring PKR activity through eIF2α phosphorylation; however, a more definitive way of showing PKR involvement would be to study PKR directly. Cloning PKR from rainbow trout could be accomplished firstly by designing primers based from conserved regions between known fish PKR-like sequences and mammalian sequences. Care must be taken to choose regions that are conserved and specific to PKR, as many proteins will have DRBM and kinase domains. These primers could be used for a PCR-based method to identify PKR from rainbow trout cDNA. After obtaining and sequencing a fragment of PKR, 5' and 3' RACE PCR could be used to obtain a full length transcript of PKR. The promoter region would be valuable for understanding PKR expression control and would be identified using genomic DNA instead of cDNA.

Preliminary experiments were performed by aligning human, mouse and rat PKR sequences with the carp PKR-like sequence in order to develop appropriate primers. Two forward primers were used, the first, ZA2F, was based on the carp Zα2 domain sequence, while the second, DSRBMF, was specific to one of the two human dsRNA binding motif sequences. The reverse primer, EIFDR, was specific to the carp eIF2V domain sequence. All three primers associated with unknown rainbow trout ESTs, found using the GRASP EST database (http://web.uvic.ca/cbr/grasp/). Using ZA2F and EIFDR primers and RTS11 cDNA, RT-PCR was performed and a ~500bp fragment was amplified, which was later cloned and sequenced. A

region of the resulting sequence showed strong similarity to two unknown cDNAs from *Danio rerio* and *Tetraodon nigroviridis* as well as *Rattus norvegicus* staufen (see Appendix B). Staufen is a dsRNA binding protein associated with mRNA localization and translation regulation (Saunders and Barber, 2003). In fish, staufen has only been identified in zebrafish (Bateman et al., 2004). More research is necessary to find the full sequence of this gene and to identify and characterize the putative dsRNA binding protein it encodes.

Clearly, this study along with many others have introduced new exciting pathways to investigate in order to more fully understand antiviral mechanisms in fish. The avenues suggested above will not only shed light on the antiviral host response, but also give insight into general cellular and viral mechanisms. Ultimately, this knowledge will contribute to not only fish health maintenance but to a better understanding of the intricate dance between host and pathogen.

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APPENDIX A

Table 1. Examples of homotypic aggregation (HA) quantification methods. Eight different methods of reporting HA found in the literature are listed. The method used in this thesis is a modification of the quantification by observation method by Yamauchi et al., 2002, see chapter 4 for a detailed description.

Aggregation Quantification Technique	Cons	Reference
1) Quantification by observation		
• Before scoring aggregation, well contents gently stirred • Aggregation scored from 0-5+. 0=no aggregations, 1+ <10% of the cells in aggregates, 2+ >10%<50%, 3+ >50%, 4+ >50% in moderate size aggregate clusters, 5+ >50% in large aggregate clusters • Scoring done independently by two individuals and averaged for final aggregation score.	Subjective, difficult to determine % of cells in aggregates	(Ainsworth et al., 1996)
• Scored aggregation by size of aggregates, - no aggregates, 1+ 1-5 cells/aggregate, 2+ 6-10 cells/aggregate, 3+ 10-15 cells/aggregate, 4+ >15 cells/aggregate • Also included sample pictures	Subjective, easier to quantify compared to % of cells in aggregates	(Kasinrerk et al., 1999)
• Count numbers of aggregates per 25X field as observed by more than one independent observer • Interobserver variance being less than 10%.	No allowance for size of aggregate	(Mentzer et al., 1986)
• Count aggregates consisting of 12 or more cells at 4X magnification • Also reported the number of cells/aggregate	Hard to determine >12 cell aggregates by eye	(Weeks and Iuorno, 1996)
• Digital pictures taken of aggregates at specific magnifications • Photoshop used to quantify size of aggregates	Measures size but not quantity of aggregates Useful if combined with manual counting of aggregates	(Yamauchi et al., 2002)
2) Observation without quantification		
• scoring aggregation as + or – • included corresponding pictures	Not quantitative	(Fernandes et al., 1999)
• Pictures of aggregates, no scoring	Not quantitative	(Dialynas et al., 1997)
3) Automated Quantification		
• Flow cytometry to measure aggregates	Cost	(Neelamegham et al., 2000)

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APPENDIX B

The known mouse, rat, human PKR sequences were aligned with the carp PKR-like sequence. Alignment was performed using Clustal W (1.83).

```
Mouse           ------------------------------------------------------------
Rat             ------------------------------------------------------------
Human           GCGGCGGCGGCGGCGCAGTTTGCTCATACTTTGTGACTTGCGGTCACAGTGGCATTCAGC 60
Carp            ------------------------------------------------------------


Mouse           ------------------------------------------------------------
Rat             ------------------------------------------------------------
Human           TCCACACTTGGTAGAACCACAGGCACGACAAGCATAGAAACATCCTAAACAATCTTCATC 120
Carp            ------------------------------------------------------------


Mouse           ------------------------------------------------------------
Rat             ------------------------------------------------------------
Human           GAGGCATCGAGGTCCATCCCAATAAAAATCAGGAGACCCTGGCTATCATAGACCTTAGTC 180
Carp            ------------------------------------------------------------


Mouse           -------------------------------------------------GAAGGCGGAGTC 12
Rat             ------------------------------------------------------------
Human           TTCGCTGGTATACTCGCTGTCTGTCAACCAGCGGTTGACTTTTTTTAAGCCTTCTTTTTT 240
Carp            ------------------------------------------------------------


Mouse           CGCCGGGAAAACGAAACAGAAGAGAACCGGCCAGGC---CCGGACTTCCATGGGCAGCAG 69
Rat             ----------------------GAACTAGCCTGAC---CTGGACTTGCCCGG----CAG 30
Human           CTCTTTTACCAGTTTCTGGAGCAAATTCAGTTTGCCTTCCTGGATTTGTAAAT--TGTAA 298
Carp            ----------------------------------------------------GGGTGTCTAG 10
                                                                       *

Mouse           CAGCGGCAGGGAACGGAGGGCGAATAGATTTCA---GAGCCTGCACCTGAAGTACAATTC 126
Rat             AAGCGACAGTGGTCGGAGGGCGACGAGATTTTA---GGGGCAGCACCTGAAGCACAATTC 87
Human           TGACCTCAAAACTTTAGCAGTTCTTCCATCTGACTCAGGTTTGCTTCTCTGGCGGTCTTC 358
Carp            ACCGACCAGAGAAGTTGCAGTACAAGAATCTGA------ACTGTATTAAAAACAT--TTT 62
                      **          *       ** * *         *             **

Mouse           GAATCCTGCTCCAGGGAGCGAGCCACTGTCCGGATCCA--------GAAACT------TT 172
Rat             GAGTCCTGCGCCAGGGCACGGGCCATTGTCCGGATCCA--------GAAAGT------CG 133
Human           AGAATCAACATCCACACTTCCGTGATTATCTGCGTGCATTTTGGACAAAGCTTCCAACCA 418
Carp            GGA--CAGAAACAAAATGTCTGCCGAAACTCAAATGGA-----GAGGAAGATCA----TT 111
                     *     *         *            *  *         **  *

Mouse           GGCCACTGGGAGGAAAAATGGCCAGTGATA---CCCCAGGTTTCTACATGGA-CAAACTT 228
Rat             GTCACCGGGGAAGAAAAATGGCCAGTGATA---CACCAGGTTTCTACGTGGA-CAAACTT 189
Human           GGATACGGGAAGAAGAAATGGCTGGTGATCTTTCAGCAGGTTTCTTCATGGA-GGAACTT 477
Carp            GATTTCTTGAGACAAAATGGGAAAAGCATAGCTTTGACAATTGCTAAAGAAATCGGACTT 171
                *    *  *    * **  **      **           ** **      *    ****

Mouse           AATAAATACCGCCAGATGCACGGAGTAGCCATTACGTATAAAGAACTTAGTACTTCGGGA 288
Rat             AACAAATACTCCCAGATACACAAAGTAAAGATTATATATAAAGAAATTTCTGTTACAGGA 249
Human           AATACATACCGTCAGAAGCAGGGAGTAGTACTTAAATATCAAGAACTGCCTAATTCAGGA 537
Carp            GACAAAT-CCACCGTGAACAGACATTTGT-ACAACCTACAGAGATCAAACCAGGTGTTTA 229
                 * * ** *   *     **   * *       *  **   ***              *

Mouse           CCTCCACATGACAGAAGGTTTACATTTCAAGTTTTAATAGATGAGAAGGAATTTCCAGAA 348
Rat             CCTCCACACGACAGAAGGTTTACATTTCAAGTTATAATAGAAGAGAGGGAATTTCCAGAA 309
Human           CCTCCACATGATAGGAGGTTTACATTTCAAGTTATAATAGATGGAAGAGAATTTCCAGAA 597
Carp            ACTCTA----ACGAAAAGCCTCCTGTTTGGGATCTAATGGA----------------GAA 269
                 *** *    *    * *  * *  **   * * **** **                ***

Mouse           GCCAAAGGTAGATCAAAGCAGGAGGCAAGAAACGCTGCAGCCAAATTAGCTGTTGATATA 408
Rat             GGTGAAGGTAGATCAAAGCAGGAGGCGAAAAATAATGCTGCCAAATTAGCTGTCGAAATA 369
Human           GGTGAAGGTAGATCAAAGAAGGAAGCAAAAAATGCCGCAGCCAAATTAGCTGTTGAGATA 657
Carp            GACGAATG-GGATCAAACAAACAGTCAA--AACACCAGAGCAAAAGTCTCTGACGA---- 322
                *   ** *  *******  *  *  * *  **       ** *** *  ***  **
                    DSRBMF primer
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Mouse           CTTGATAACGAAAACAAGGTGGATTGTC-----------ACACGAGTGC-ATCTGAGCAA 456
Rat             CTTGATAATGAAAACAAGGTGGATAGTC-----------ACACGGATGC-TTCTGAACAA 417
Human           CTTAATAAGGAAAAGAAGGCAGTTAGTCCTTTATTATTGACAACAACGAATTCTTCAGAA 717
Carp            --CGACAGCAGAAACATG-TGAAGAGAA-----------ACACGTAAGGGATCTGCTGAA 368
                    * *    *** * *       *             ***     *   ***    **

Mouse           GGCTTGTTCGTTGGTAACTACATAGGCCTTGTCAATAGCTTTGCCCAGAAGAAAAAGCTG 516
Rat             GGTTTAATAGAGGGGAACTACATTGGCCTTGTCAATTCTTTTGCCCAGAAGGAAAATCTG 477
Human           GGATTATCCATGGGGAATTACATAGGCCTTATCAATAGAATTGCCCAGAAGAAAAGACTA 777
Carp            A-TCAGGAGGTTTAAAAGCCTGTCAGATCGCTAAAGACTTGGGACAACCAAGAAAAGCCG 427
                               **     *  *     * **       * * *  *  ***  *

Mouse           TCTGTAAATTA-TGAACAGTGTGAGCCCAACTCTGAGTTGCCTCAAAGATTTATTTGTAA 575
Rat             CCTGTAAATTT-TGAACTGTGTGACCCCGACTCCCAATTGCCTCACAGATTTATTTGTAA 536
Human           ACTGTAAATTA-TGAACAGTGTGCATCGGGGGTGCATGGGCCAGAAGGATTTCATTATAA 836
Carp            TAAACAAACAGCTGTACAGTATGATGCAGACGGGTAAAG----TAAAGA----------A 473
                     ***    ** ** ** **   *       *        *  **          *
                               ZA2F primer

Mouse           ATGCAAAATTGGGCAGACAATGTATGGTACTGGTTCAGGTGTCACCAAACAGGAGGCAAA 635
Rat             ATGCAAAATCGGGCAGACTACGTATGGTACTGGTTTCGGTGCTAACAAAAAGGAGGCAAA 596
Human           ATGCAAAATGGGACAGAAAGAATATAGTATTGGTACAGGTTCTACTAAACAGGAAGCAAA 896
Carp            ATGTGAAAT-----AAGTAGCTTGTGGC-----TTCTGG----AAGGCGAGGAGAGCAAT 519
                ***  ****     *       * * *      *   **    *       *   ****

Mouse           GCAGTTGGCTGCGAAAGAAGCCTATC---AGAAGCTGTTAAAGAGCCCGCC---GAAAAC 689
Rat             GCAGTTGGCTGCCAAAAATGCGTATC---AGAAGCTGTCAGAGAA----------AAGCC 643
Human           ACAATTGGCCGCTAAACTTGCATATCTTCAGATATTATCAGAAGAAACCTCAGTGAAATC 956
Carp            GAAAGTCACTCCCAAGAGAGTG-ATC---ATAGACTG------------------GGGTC 557
                  *  *  *  * **    *   ***   * *   *                      *

Mouse           TGCCGGAACATCCTCTAGCGTTGTCACATCTACATTCAGTGGCTTTTCCAGCAGCTCGTC 749
Rat             CATCGAAAA-----CTGGCTTTGTCACATCTTTATCCAGTGACTTTTCCAGCAGCTCGTC 698
Human           TGACTACCTGTCCTCTGGTTCTTTTGCTACTACGT---GTGAGTCCCAAAGCAACTCTTT 1013
Carp            AGTCGCAG--------GGTTGTCTCAAAGTTTTGAT--GTGA---TCACAAAGCTTGGTG 604
                   *             *   * *      *       ***        *     *  *

Mouse           TATGACAAGT-AATGGTGTTTCCCAGTCAGCACCTGGAAGTTTTTCCTCAGAGAACGTGT 808
Rat             TATAACAAGT-AACTCTGCTTCTCAGTCAGCATCTGGAAGGGATTTC---GAGGACATAT 754
Human           AGTGACCAGC-ACACTCGCTTCTGAATCATCATCTGAAGGTGACTTCTCAGCAGATACAT 1072
Carp            AAGGAGGATTTGGCTGTGTTTTTAAAGTAAAGCATAAATTTGATGGC----AAGATCTAC 660
                    *  *          * **   *   *     *  *        *       *

Mouse           ---------------------------------------------TTACGAACGGTCTCG 823
Rat             ---------------------------------------------TTATGAATGGTCTCA 769
Human           CAGAGATAAATTCTAACAGTGACAGTTTAAACAGTTCTTCGTTGCTTATGAATGGTCTCA 1132
Carp            GCTG-----------------------------------------TAAAGAAAGTCGTCT 679
                                                             * * *** *   **

Mouse           GAGAAAATAAAAGGAAATCAGGAGTAAAAGTATCCCCTGA------------TGATGTGC 871
Rat             GAGAAAA---AAGGAAATCAGGAGTAAAAGTGCCATCTGA------------TGACGTGC 814
Human           GAAATAATCAAAGGAAGGCAAAAAGATCTTTGGCACCCAGATTTGACCTTCCTGACATGA 1192
Carp            TAACTGGGGAAGCTGATTCCGAGGTGAAGGCATTGGCCAG-------------ACTAGAC 726
                 *        *    *  *                 *

Mouse           AAAGAAATAAATATACCTTGGACGCCAGGTTTAACAGCGATTTTGAAGACATAGAAGAAA 931
Rat             TAAGAAATAAATATACCTTGGACGACAGGTTTAGCAAAGATTTTGAAGACATAGAAGAAA 874
Human           AAGAAACAAAGTATACTGTGGACAAGAGGTTTGGCATGGATTTTAAAGAAATAGAATTAA 1252
Carp            CATCCAAACATAGTGCGCTACATTACATGTTGGCC--GGATTCTGAGAGCTGCACATCAA 784
                 *   *   *   * *  *  *    * ***   *   **** * *         *  **

Mouse           TTGGCTTAGGTGGATTTGGTCAAGTTTTCAAAGCGAAACACAGAATTGATGGAAAGAGAT 991
Rat             TTGGCTCGGGTGGATTTGGCCAAGTTTTCAAAGCAAAACACAGAATCGATGGAAAGACGT 934
Human           TTGGCTCAGGTGGATTTGGCCAAGTTTTCAAAGCAAAACACAGAATTGACGGAAAGACTT 1312
Carp            ---ACCAAGACAGA---AACCAAGTGTCCAA--CACATCAGGTTCTTCATCATGTGGAGT 836
                    *   *   **      ***** * ***  *  * **     *  *      *   *

Mouse           ACGCTATTAAGCGCGTTAAATATAACACGGAGAAGGCGGAGCACGAAGTACAAGCGCTGG 1051
Rat             ATGCTATTAAGCGCATTACATATAACACCAAGAAGGCAAAGCGTGAAGTACAAGCACTAG 994
Human           ACGTTATTAAACGTGTTAAATATAATAACGAGAAGGCGGAGCGTGAAGTAAAAGCATTGG 1372
```

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Carp            G-ACCTTTGATAGAGCTGGCTGTGAAGAGAGGAATGATGAAGACGA--TGAAGACGATGA 893
                      ** *  *   *   * * *      *** *   *    **  *  *  *  *

Mouse           CAGAACTCAATCACGTCAACATTGTCCAATACCATAGTTGTTGGGAGGGAGT------TG 1105
Rat             CAGAACTCAATCACGCCAACATTGTTCAATACCGAGTTTGTTGGGAGGGAGAGGACTATG 1054
Human           CAAAACTTGATCATGTAAATATTGTTCACTACAATGGCTGTTGGGATGGATTTGATTATG 1432
Carp            T-GATGACGATGATGTCAGTGATGT-CACATCAAGAATGGAAAGTCTGGGTTCGACAGCA 951
                   *     ** * *  *    *** **   *       *   *   **

Mouse           ACTATGA---------------TCCTGAGCACAGCAT------------GA---GTGATA 1135
Rat             ACTATGA---------------TCCCGAGAACAGCAC------------AAACGGTGACA 1087
Human           ATCCTGAGACCAGTGATGATTCTCTTGAGAGCAGTGATTATGATCCTGAGAACAGCAAAA 1492
Carp            GAATTAGCATCTG--------CTGCTGGGCCCTCCG-------------GAAACCTGGAC 990
                    *                *   * *  *                  *

Mouse           CAAGTC---GATACAAAACCCGGTGCCTCTTTATTCAAATGGAATTCTGTGATAAAGGAA 1192
Rat             CAAGTC---GATACAAGACCCGGTGCCTCTTTATCCAAATGGAATTCTGTGATAAAGGAA 1144
Human           ATAGTTCAAGGTCAAAGACTAAGTGCCTTTTCATCCAAATGGAATTCTGTGATAAAGGGA 1552
Carp            CCATTA--AATCACAGCAGGATGTATTTGTTTATTCAGATGGAGTTCTGTGAGGGAGGAA 1048
                  * *         *  *    **   * ** ** ** ***** ********   *** *
                                                          EIFDR primer

Mouse           CTTTGGAGCAATGGATGAGAAACAGAAATCA---GAGTAAAGTGGACAAAGCTTTGA--- 1246
Rat             CTCTGCAGCAGTGGTTGGAAAAGAGAAATCG---GAGTCAAGAGGACAAGGCTTTGG--- 1198
Human           CCTTGGAACAATGGATTGAAAAAAGAAGAGG---CGAGAAACTAGACAAAGTTTTGG--- 1606
Carp            CACTGACCACGTGGATACGGGCGAGAAATCGTATGAACAAACAGAGAATCGCGGTGGAAA 1108
                *  **      *** *       ****            **      *  *   **

Mouse           TTTTGGACTTATATGAACAAATCGTGACCGGAGTGGAGTATATACACTCGAAAGGGTTAA 1306
Rat             TTTTGGAGTTATTTGAACAAATAGTGACAGGAGTGGATTATATACACTCGAAAGGTTTAA 1258
Human           CTTTGGAACTCTTTGAACAAATAACAAAAGGGGTGGATTATATACATTCAAAAAAATTAA 1666
Carp            TCCATAAAATATTTTATGAAATTGTCAGTGGAGTGGAATACATCCATTCAAACAGTCTCA 1168
                      *  * * * *  ****    *  ** ***** ** ** ** ** **     * *

Mouse           TTCACAGAGATCTTAAGCCAGGTAATATATTTTTAGTAGATGAAAGACACA-TTAAGATC 1365
Rat             TTCATAGAGACCTTAAGCCAGGTAATATATTTTTAGTGGATGAAAAACACA-TTAAGATC 1317
Human           TTCATAGAGATCTTAAGCCAAGTAATATATTCTTAGTAGATACAAAACAAG-TAAAGATT 1725
Carp            TCCACAGAGACTTGAAGCCTGATAACATATTGTTTG-GCATGGATGGCAAAGTGAAGATC 1227
                * ** *****  * *****   *** ***** ** *   **  *   **   * *****

Mouse           GGAGACTTTGGCCTTGCAACAGCCCTGGAAAATGATGGAAAATCCC---------GAACA 1416
Rat             GGAGACTTTGGCCTTGCAACAGCCCTGGAAAATGATGGAAATCCTC---------GAACA 1368
Human           GGAGACTTTGGACTTGTAACATCTCTGAAAAATGATGGAAAG---C---------GAACA 1773
Carp            GGAGACTTTGGGCTGGTGGCGGCACAGACCAATCACAGCGGTGGTCCTATAGAGAGGACA 1287
                *********** ** *   *  * * *   *** *  *       *         * ***

Mouse           AGGAGAACAGGAACTCTTCAATACATGAGTCCAGAACA---GTTATTTTTAAAGCACTAT 1473
Rat             AAGTATACAGGAACTCCTCAATACATGAGTCCAGAACAAAAGTCATCGTTAGTGGAATAT 1428
Human           AGGAGTAAGGGAACTTTGCGATACATGAGCCCAGAACA---GATTTCTTCGCAAGACTAT 1830
Carp            AAGAGAAGAGGAACACTGCAATATATGAGTCCTGAACA---GGAAAATAAGAGGAATTAT 1344
                * *   *  *****    * *** ***** ** *****   *             * ***

Mouse           GGAAAAGAAGTGGACATCTTTGCTTTGGGCCTTATTCTAGCTGAACTTCTTCACACGTGC 1533
Rat             GGAAAGGAAGTGGACATCTTTGCTTTGGGCCTTATTCTAGCTGAACTTCTTCACATATGC 1488
Human           GGAAAGGAAGTGGACCTCTACGCTTTGGGGCTAATTCTTGCTGAACTTCTTCATGTATGT 1890
Carp            GATGAAAAAACAGACATTTTCCCTCTTGGACTCATATGGTTTGAGATGCTTTGGAAATTG 1404
                *   *  **   *** * *   ** * ** ** **      ***  * ***      *

Mouse           TTCACGGAGTCAGAGAAAATAAAGTTTTTCGAAAGTCTAAGAAAAGGCGACTTCTCTAAT 1593
Rat             AAAACTGATTCAGAGAAAATAGAGTTTTTCCAACTTCTAAGAAATGGCATCTTCTCCGAT 1548
Human           GACACTGCTTTTGAAACATCAAAGTTTTTCACAGACCTACGGGATGGCATCATCTCA--- 1947
Carp            TCCACCGGTATGGAGAGAGCAGAGCTGTTGAAAGATCTGAGAAATCAAAGGTTTCCGGAA 1464
                   ** *     ** * *  * ** * **   *   **  *  *        *  *

Mouse           GATATATTCGACAA------CAAAGAAAAAAGCCTTCTAAAAAAACTACTCTCAGAGAAA 1647
Rat             GATATTTTCGACAA------CAAGGAAAAAAGCCTTCTACAGAAATTACTCTCAAGTAAA 1602
Human           GATATATTTGATAA------AAAAGAAAAAACTCTTCTACAGAAATTACTCTCAAAGAAA 2001
Carp            GGTTTTTGTGACAGTTATCCAACTGAAAATAAATTCATCGAGAAGATGCTGTCCTTTGCA 1524
                * * * *  ** *        *  ***** *   *  *  * **  * ** **      *

Mouse           CCCAAGGACCGACCT-------GAGACATCTGA---AATCCTGAAGACCTTGGCTGAATG 1697
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Rat             CCCAGGGAACGACCC-------AATACGTCTGA---AATCCTGAAGACTTTGGCTGAGTG 1652
Human           CCTGAGGATCGACCT-------AACACATCTGA---AATACTAAGGACCTTGACTGTGTG 2051
Carp            CCGGAGGACAGGCCACCTGCAAAAGACATCAAAGAAAAACTTGAGAAGTTTTTCTCTCTG 1584
                **   ***  * **         * ** **  *   **   * *  *  **  **   **

Mouse           GAGGAACATCTCAGAGAAAAAGAAAAGAAACACATGTTAGGGCCTTTCTGAGAAAACATT 1757
Rat             GAAGAACATCTCGGAGAAAAAGAAAAGAAACACGTGTTAGGGCTTTT------------- 1699
Human           GAAGAAAAGCCCAGAGAAAAATGAACGACACACATGTTAGAGCCCTT------------- 2098
Carp            GATGAGGATCTGTTAAGCCAGAAAACGAT-------TTGAGATTTTTGT------------ 1626
                ** **  * *    *    *   ** **        **       **

Mouse           CCTCTGCCGTGGTTTTCCTTTAACGATCTGCAGTCTGAGG-GGAGTATCAGTGAATATTA 1816
Rat             -------------------------AACTGCAGTCTGAAGTGGAATGTCAGAGAACAATG 1734
Human           ----------------------------------CTGAAAAAGTAT-CCTGCTTCTGATA 2123
Carp            --------------------------------ATTTAAACTGCCACAGTAGTTTGGCAGA 1654
                                               * *           *

Mouse           TCCTTCTTTTCTTAATACCACTCTCCCAGACAGGTTTTGGTTAGGGTGACCCACAGACAT 1876
Rat             AACTTCTTCTCTTAA---CACTCTCCCAGACAGGTTTTGGTAAGGGTGACCCAAAGACAT 1791
Human           TGCAGTTTTCCTTAA----ATTATCTAAAA-----TCTGCTAGGGAATATCAATAGATAT 2174
Carp            GTCGACTCAAAATAA------TGTTTTA--------CCGCAGAGTACTTCTGACAGGTGT 1700
                  *   *     ***      * *   *          *    *        * **   *

Mouse           TGTATTTATTAGGCTATGAAAAAGTATGCCCATTTCCTCAATTGTTAATTGCTGGGCCTG 1936
Rat             TGTATTTATC-GGCTATGAACAAGTATGCCCGTTTCCTCAATTGTTAATTGTCGGACCTG 1850
Human           T-TACCTTTTATTTTA---------ATGT---TTCCTTTAATTTTTTACTATTTTTACTA 2221
Carp            TCTGATGATT---------------TTATCGATTTATTCATGATTTAATGAACTGTTTAA 1745
                * *     *                *     **   * *    ** *

Mouse           TGGCTGGCTAGCTAGCCAAATATGTAAATGCTTGTTTCTCGTCTGCCCAAAGAGAAAGGC 1996
Rat             TGGCTGGCTAGCCAAATATGGAAATGTTTGTTTCTTTCTCGTCTGCCCGAAGAGAAAGCC 1910
Human           -ATCTTTCTGCAGAAACAGAAAGGT-TTTCTTCTTTTTGCTTCAAAAACATTCTTACATT 2279
Carp            TGATCTCTCATTAAGGTGAAAATAT--AAAATCATAAAATGTTGAGAATAACTGTCAGCT 1803
                             *       *  *      *  *      *       *

Mouse           AGGCTCCTGTGTGGGAAGTCACAGAGCCCCCAAAGCCAACTGGATGAGGAAGGACTCTGG 2056
Rat             AAGCCGCGGTGGAGAAGGTCACTTAACCCCCAATGCTAACTG--------AAGGCCTAAGG 1963
Human           TTACTTTTTCCTGGCTCATCTCTTTATTCTTTTTTTTTTTTTTAAAGACAGAGTCTCGCT 2339
Carp            TTTCTAGGATTTCTTCTAATATATTTTTTAAAATGTCAAAAGTTGCATTTCAGTCTCATT 1863
                   *                                                * **

Mouse           CTTTTGGC-ATAAAAAAGAGCTGGTAGTCAGAGCTGGGGCAGAAGGTCCTGCAGACAGAC 2115
Rat             CTTTTGAC-ATACTAAAGAGCTGGCAGCCAGAGCTGGGAGAGAAGAACCTGCAGACAGGC 2022
Human           CTGTTGCCCAGGCTGGAGTGCAATGACACAGTCTTGGCTCACTGCAACTT-CTGCCTCTT 2398
Carp            TTGTTTTC-ATCTTAAAATACATTTTAATGACGTTGTGTGGCTACTTTTTACAA--AACC 1920
                 * **  * *      *   *             **             * *

Mouse           AGACAGACAGACAGACAGACAGACAGACAGACAGAGACACAAAGACAT-GGACTAGAATG 2174
Rat             AGGCAGA----------------------------GACACAAAGACAT-GGACTAGAATG 2053
Human           GGGTTCA--------------------------AGTGATTCTCCTGCCTCAGCCTCCTGAG 2433
Carp            AACCATG------------------------------AACGTAATTTCACAACATTACTGTA 1952
                                                     *       *      *

Mouse           GAGGAGGGAGGGAGGAAGGGAGGGAGGGAGAGAGAGAGAGAGAAAGAAAGAGAGAGAGAC 2234
Rat             AAACAGGAAGGTAG--------------AGAGATTGAGAGACAGAGCCACATGGAGAGAC 2099
Human           TAGCTGGATTACAG-----------------GCATGTGCCACCCACCCAACTAATTTTTG 2476
Carp            ATGTTGGCTGCAAA-----------------ATTAAGTTGTATTTATAATTGCAACTAA 1994
                     **     *                        *         *

Mouse           CACATGGAGAGACAAAATGGCTTAAGTTAGCTGGGCTACCTGAGAGACTGTCCCAGAAAA 2294
Rat             GAAATGGCAGAGTAGTA-GGCTTAAGTTAGATGAGCTAACTGAGAGACTGCCCCAGAAAA 2158
Human           TGTTTTTAATAAAGACAGGGTTTCACCATGTTGGCCAGGCTGGTCTCAAACTCCTGACCT 2536
Carp            AGTATAATGTTTCTCAA-AGTTACAATTGGTTCGGTTACAGTGTAACCAGACTGCAACCA 2053
                    *           *  * *  *    * *                       *

Mouse           CAGGCCAACAACCTTCCTTATGCTATATAGATGTCTCAGTGTCTTTATCATTAAACACCA 2354
Rat             CAGGCCAACAGCCTTA-------TACACAGATGTCTCAGTGTCT--ATCATTAAACTCCA 2209
Human           CAAGTAATCCACCT----------GCCTCGGCCTCCCAAAGTGC------TGGGATTACA 2580
Carp            AAATACAACACTGTGTAATG---TGAAGAAATGTTGTGCTGGCT------GGGAACATT- 2103
                 *    * *    *                  *      *           *
```

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Mouse           AGCAGGACTGCTAAAAACTCTGCAATAGGGTTTTTTTTTTTCCTGTTACTTCAAAAGCAA 2414
Rat             AGCAGGATCAATGAAGGCCCTACAATAGG---TTTTTTTTTCTTGTTACTTCAAAAACAA 2266
Human           GGGATGAGCCACCGCG-CCCAGCCTCA-----TCTCTTTGTTCTAAAGATGGAAAAACCA 2634
Carp            -GCTGCTCCTGTTGATTTTTGACAAATG----TACTATGTATGTGGAATAAAACAGACAG 2158
                 *                   *         *    *     *        * *  *

Mouse           TCTTACAAAGTTATTTTTTTGACAATTCCATACATGCATTGTGTTCTGATCCCACTCTGA 2474
Rat             TCTTACAAAGTTATTTTCTTGAGAGTTCCATATGTGTATTGTATCCTGATCACACTCCAA 2326
Human           CCCC-CAAATTTTCTTTTTATACTATTA-ATGAATCAATCA-ATTCATATCTATTTATTA 2691
Carp            AAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA---------------------------- 2192
                      ***           *       *

Mouse           ACCCTCTGCCATTCATGCCTTGTCTGTCATGTGAACTGTTGCCTCTGAATGTGGGGGTCC 2534
Rat             ACCTTCTGCTGTTCATGCCTTATC----ATGTGAACTAT-ACCTCTGAACGTGGGAGTCC 2381
Human           AATTTCTACCGCTTTTAGGCCAAA--------AAAATGTAAGATCGTTCTCTGC---CTC 2740
Carp            ------------------------------------------------------------

Mouse           AAATTAACCCTCTGCCCTTGAGTG--GCTTCTCTCAGGTAGTG-----ATTGTGATGAGA 2587
Rat             AAATTGTCCATCTGCTCCTTAACGTTACTTCTCATAGGTAGTTTAGTCGTTGTGATGAGA 2441
Human           ACATAGCTTACAAGCCAGCTGGAGAAATAT------GGTACTC-----ATTAAAAAAAAA 2789
Carp            ------------------------------------------------------------

Mouse           AAAGTAATGAGATGCTGGCAAAGATGTGCAGAAAGAAGAACACTTCTCCACTGCTGGTAG 2647
Rat             AAAGTAATGAAATGGTACCAGAAATGGGGTTTTGTCGCAATAAACCTACTGTGC------ 2495
Human           AAAAAAGTGATGTACAACC------------------------------------------ 2808
Carp            ------------------------------------------------------------

Mouse           GATTGCAAGCTGGTACAACCACCCTGGAAATCAGACTGGAGGTTCCTCAGAAACACAGTA 2707
Rat             GGTTTC---TTGG-------ACTTTCAGGACTGGTTTGTGGG------AAGAATGTGGAA 2539
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CTACCTGAGGACCCAACAATACCACTACTGGTCATATACCCAGAAG--ATGGTCCAACAT 2765
Rat             AAGTTTGAAG---CAGCAG-------GCTAGAGAAGTCCTCAGATGGTATACGTCAATCT 2589
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GTAATATGGACACATGCGCCACTATGTTCATAGTAGCCTTATTTATAATAGCCAGGAGCT 2825
Rat             TAATTCTGGTAGCTCAGAAGTCTTTGCCAAAAAGGAACCCATTGGGAATTGG-ACTAGAT 2648
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GGAAAGAACCCAGATGTCCCTCAG-CAGAGGAATGGATACAGAAAATGTGGCACATTTAC 2884
Rat             GCCAGGA---CATTTGTGTACAAGTCAAAAGACTGGTGAGGGAAATTTCAAGACATTCAG 2705
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           ACAATGGAGTACTACTCAGCTATTAAAAATGAATTCATGAAATTCTTAGACAAAT-GGAT 2943
Rat             ACTGTGGCATGGTGGTTATCTCCTTCTCATAGCT----AGAATTATAGAGCAAATTAGGA 2761
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GGATCTGGAGGATATCATCTTGAGTGAGGTAACCCAATCGCAAAAGAACACACATGATAT 3003
Rat             AGGAGTGAGGGGTAAGA-----AGTGAGAGAAC---------AAAGATCTGGGAATGAGT 2807
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GCACTCACTGATAAGTGGATATTAGCCCAAAAGCTCCAAATAACCAAGATACAATTCACA 3063
Rat             GCCTTCAAAGACGAG-----GTCGGTGCAAAGCTGTTAAA----------GCGATTAGTG 2852
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------
```

```
Mouse           GACTACATGAAGCTCAAGAAGAAGGAAGACCAAAGTGTGGGTGCTTTGGTCCCTCTTAGA 3123
Rat             CACT-----------------GAGGGAAGCC----ATGTACTTTCCATTGCTGCCATTATA 2892
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           AGGGGAACAAAGTACTCACAGGAGCAAATATGGAGATAGAGTGTAGAGCAGAGACTGAAG 3183
Rat             GGAACAAGACTACAGCCACGGGCG---------------GCTGCACAGCATGGA---AAG 2934
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GAAAGGCCATCCAGAGACTGTCCCATATACAGAGACTGGGAATTCATCCCATACACAGTT 3243
Rat             TAAAAGCCCTTTAAAGGTC-TTTCGTGTCAAAAG-----------TTCCA---GCAGGT 2978
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           ACCAAACCCAGACACTATTGTGGATGCCAAGACATTCATGCTGACAGGAGCCTGATATGG 3303
Rat             GCCA----CAGCCACTCCAGTGGA---------GTCTGTG-TGACAGGAAGCTCTTGTGC 3024
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CTGTCTCCTGAGAGGTCCTGCCAGAGCCTTACAATACAGAGACTGATGCTCACAGCCAAC 3363
Rat             TTCGTTTCTATGCAG----AACAGAGGCCTG---TGCCTGGAATG--GCTTGTCCTC--- 3072
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CACTGGACTGAGTGTGGGGTCCCCAATAGAGGAGTTAGAGAAAGGACTGAAGGAGTTGAA 3423
Rat             --CTGGAGGTCCTGGCAGTCTCCCTGCAAAAAGATGGTGACCAGGGTCTATGCTTTCAGT 3130
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GGGGTTTGCAACCCCATAAGAACAACAATATCAACCAAGCAGACCCCCCAGAGCTCCCAG 3483
Rat             GGAATTGCCTTCTGTGTTCTAGCCTTGGTG--ATCTTAGCGA---TTTTAAGCCTGTCAA 3185
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           TGACTAAGCCATCAACCAAGGAGTACACATGGCTC--CAGCTGCATATGTAGCAGAGGAT 3541
Rat             CAATA----TCTGATTTTAGGAATATAGCAGGGTGGGCAGATGATCATGTATCTTTAGAT 3241
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GGCCTTGTCATGTATCAAAAGGAGGAGAGGTCCTTGGTCCTATGAAGGTGC-GATAGATG 3600
Rat             GTC----TCAGGCACTGAAATAAATAAATGTCTC--ACACCATCATGGTCCTGATGAACT 3295
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CCCCAG-TATAGGGGAATCAAGGGCAG-ATAGGTGGGTTGGAGGAACACCCTCATAGAAG 3658
Rat             CAATGGCTGCAATGAATTCAACTGCTGTATCCATTAGTTCCCAGAAAATAGGAGAGAAAT 3355
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CAGGGGGAGTAAGGAAGGATATGGGGATTTCTGGGAGGGGTGGAAACTAGGAAAGGGG-G 3717
Rat             TAATTGCAGTTACTAAGAACATGACTGTTCCAGAAAAATCTTTCCCCCCTTTTGGGAAAG 3415
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           TAACATTTGAAATG-TAAATAAAGAAAATATCCAATTAAAAAAAAAAGAAAAAGAAAAAA 3776
Rat             TTTTGTTTAGCATGATTCAGAATAGTAATGATTCTTTTAAAAAAGATCATAGACAACTTC 3475
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------
```

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Mouse           GAAAAGAATAGTAATAAAATGGTACAGGAAGTAGAGTTATATTGCAATAAACCTACTGTT 3836
Rat             CAACAAGTTG--AACAAACTT--------AACAGAGTTA------ACTATGGTTACTTTT 3519
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GGGCTTTCAGGACTGGTTTGTGGGAGGAATGTGAAAAAGTTTGAAGCCCCAGGTTAGAGA 3896
Rat             GCTTCCTTACTATTCTGAGGGGCCATAAATATAATAGAGTTTAAACTTT---ATTAAAA- 3575
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           AGTCCTCAAATGGTATACGTCAAACTTACTGTGGTAGCTCAAAAGTCTCCTGAGAGGCCC 3956
Rat             ------CAAA-GGTAAATGATGAATACATACCTGGTGCCCACCAGTCT------------ 3616
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           TGCTTGGAGTTAGCCTTGTAGAGGTCCAGTCTTTCCTTGTTGTTCTTTCAGACTTGCTTT 4016
Rat             -----GGCAGTATAACTGCAGTTTTCTAAACTAT---TGTTTTCAGCTGAGAATTGTTTT 3668
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           GTAGAATATTGGTAGTTACTTTGTGCCTTTGTATGCTGTAATAGTTGTTTTATAGGGCCT 4076
Rat             CTAGACTGTATGTT-CCGCTTTCCTCCTT--------GCAAGGGACATTTGGTGAGGAG- 3718
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           CACAGCTAAGAGTTTCTCG-CTGCTTCTCAAAGCACTTTGGACCTTTGCATGGAGTTGAG 4135
Rat             -ATGAATAAGAGCTAGATGATTGCCAATTATGATGTATCTGTTCTAACTGTTTAAGTCTA 3777
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           TATTAAGATTATGGGAATTTCTGAGGTGGGACTGAAAGCATTTTGCATTATGAGATGGCC 4195
Rat             TGCCAAAAA-GTAAAGCTTTTTACAACAACAC---------------------------- 3808
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           ATGAGCCAACAGAGACTTGGACACACTCCTCCACTGTCAACCGAGGCTTCTGCCAAATCT 4255
Rat             ------------------------------------------------------------
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------

Mouse           TCCCTGTCATGAAGGATTGTATCATCTGAAATTGAGTCTAAATAGATAAATAAATAAGC 4314
Rat             ------------------------------------------------------------
Human           ------------------------------------------------------------
Carp            ------------------------------------------------------------
```

Using ZA2F and EIFDR primers, a 524bp fragment was amplified:

```
TTCCTCCCTCACAGAACTCCCCCACCGCCACACGCACCATGAAGTTCTTCATGTGGGGAGGACCTGCCTCCTTCAGAACCTCAAAGTTG
ACAGGCATGTTCCTCTTCAGTGCGATCTCAAACACCTGGCTGATTTCTGATTTGTTGAGGTTCTCTTCAGTATCTTCTCCATTCATCTC
AGGCAGCTGCTGCAGCAGTATGGGCTCCTTCTGCAGAGTCTTCAGGGCCTTGGAGGCGGCGTCGTGTTTGGCGAGCTGCCGCGTACGCC
CCTTCCCGTGGAACTGCTGGcCCCCGATGTTCAGCTCCATGTGGTACAGCACCGGCCCCACTGgGGGAAACGGGTAGTAGTACTGTTGC
ATGGAGCGTTGGTAAGGCCCCGGTGCCCTGATGTTGTAGTTGAAGCTGGGTAGTCTCATCCCGGGGTAAGGGTCTATAGGCTTGTACAT
AGGCTTCATCCCTAACTTCATACAGAGAAGAAATCAGCTCCGCGAGATCTAGGCGAAGTCAGTGACTAGTGAACAGAAG
```

A region of this fragment (O.) had very high similarity to cDNAs from *Tetradon nigroviridis* (T., accession number: CR651670) and *Danio rerio* (D., BC065333), both of unknown identity, as well as the staufen RNA binding protein from *Rattus norvegicus* (R., BC101858).

```
T.              ----------------------------------------------TCTTCATGTGGGGAG 15
R.              ------------------------------------------------------------
O.              TTCCTCCCTCACAGAACTCCCCCACCGCCACACGCACCATGAAGTTCTTCATGTGGGGAG 60
D.              ----------------------------------------------TCTTCATGTGGGGAG 15


T.              GACCTGCCTCCTTCAGAACCTCAAAGTTGACAGGCATGTTCCTCTTCAGTGCGATCTCAA 75
R.              -----------------ACCTCAAAGTTGACAGGCATGTTCCTCTTCAGTGCGATCTCAA 43
O.              GACCTGCCTCCTTCAGAACCTCAAAGTTGACAGGCATGTTCCTCTTCAGTGCGATCTCAA 120
D.              GACCTGCCTCCTTCAGAACCTCAAAGTTGACAGGCATGTTCCTCTTCAGTGCGATCTCAA 75
                          ****************************************


T.              ACACCTGGCTGATTTCTGATTTGTTGAGGTTCTCTTC----------------------- 112
R.              ACACCTGGCTGATTTCTGATTTGTTGAGGTTCTCTTC----------------------- 80
O.              ACACCTGGCTGATTTCTGATTTGTTGAGGTTCTCTTCAGTATCTTCTCCATTCATCTCAG 180
D.              ACACCTGGCTGATTTCTGATTTGTT----------------------------------- 100
                ************************


T.              ------------------------------------------------------------
R.              ------------------------------------------------------------
O.              GCAGCTGCTGCAGCAGTATGGGCTCCTTCTGCAGAGTCTTCAGGGCCTTGGAGGCGGCGT 240
D.              ------------------------------------------------------------


T.              ------------------------------------------------------------
R.              ------------------------------------------------------------
O.              CGTGTTTGGCGAGCTGCCGCGTACGCCCCTTCCCGTGGAACTGCTGGCCCCCGATGTTCA 300
D.              ------------------------------------------------------------


T.              ------------------------------------------------------------
R.              ------------------------------------------------------------
O.              GCTCCATGTGGTACAGCACCGGCCCCACTGGGGGAAACGGGTAGTAGTACTGTTGCATGG 360
D.              ------------------------------------------------------------


T.              ------------------------------------------------------------
R.              ------------------------------------------------------------
O.              AGCGTTGGTAAGGCCCCGGTGCCCTGATGTTGTAGTTGAAGCTGGGTAGTCTCATCCCGG 420
D.              ------------------------------------------------------------


T.              ------------------------------------------------------------
R.              ------------------------------------------------------------
O.              GGTAAGGGTCTATAGGCTTGTACATAGGCTTCATCCCTAACTTCATACAGAGAAGAAATC 480
D.              ------------------------------------------------------------


T.              -------------------------------------------
R.              -------------------------------------------
O.              AGCTCCGCGAGATCTAGGCGAAGTCAGTGACTAGTGAACAGAAG 524
D.              -------------------------------------------
```

BLAST search E values:
Tetraodon nigroviridis E value= 8e-12
Danio rerio E value = 2e-09
Rattus norvegicus staufen E value = 5e-07