STUDIES ON HOST-PATHOGEN INTERACTIONS OF INFECTIOUS SALMON ANAEMIA VIRUS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Doctor of Philosophy

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Dr. Tomy Joseph

Charlottetown, P. E. I.

August, 2004

Your file Votre référence
ISBN: 0-612-93849-2
Our file Notre référence
ISBN: 0-612-93849-2

CONDITION OF USE

SIGNATURE

PAGE(S)

(iii) & (iv)

REMOVED

ABSTRACT

Infectious salmon anemia (ISA) virus (ISAV), an economically important pathogen in marine aquaculture has been classified in the family *Orthomyxoviridae,* genus *Isavirus.* ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon and asymptomatic infection in both farmed and feral fish. It is well documented that Atlantic salmon that recover from clinical ISA as well as subclinically infected trout and herring can transmit ISAV to healthy Atlantic salmon through cohabitation. The mechanisms of pathogenesis and persistence of ISAV are not adequately studied at the molecular level. This study investigated the host-pathogen interactions of ISAV. Major aspects of this study included the determination of mechanisms of cell death during ISAV infection of fish cells and the exploration of the novel phenomenon of antibody-mediated growth of ISAV in macrophage-like fish cell lines.

Two general pathways known to cause viral cytopathic effect (CPE) are apoptosis and necrosis. In this study, three permissive fish cell lines SHK-1, CHSE-214, and TO were used to determine if ISAV-induced CPE is due to apoptosis or necrosis. Characteristic apoptotic DNA fragmentation was observed only in ISAV-infected SHK-1 and CHSE-214 cells. The apoptosis was confirmed by use of the fragment end labeling assay. ISAV-infected TO cells did not undergo apoptosis but showed leakage of the high mobility group 1 (HMGB1) protein from the nucleus which is characteristic of cells undergoing necrosis. Infected SHK-1 cells did not show leakage of HMGB1 protein. Infection with two different strains of ISAV showed that the induction of apoptosis correlated with the appearance of CPE in SHK-1 cells. These cells did not show caspase-3-like proteolytic activity. The ISAV-induced apoptosis was however inhibited by a pan-caspase inhibitor, Z-VAD-fmk, indicating it is caspase dependent. The ISAV putative PB2 protein and translation products of RNA segment 7 specifically bound caspase-8 *in vitro* suggesting that these viral proteins may have a role in the ISAV-induced apoptosis.

To study ISAV-antibody interactions, virus neutralization (VN) was performed in the three cell lines using three strains of ISAV and rabbit or fish anti-ISAV sera. Homologous VN titres of >1:1280 in CHSE-214 cells corresponded to only ≤ 1:80 in macrophage-like fish cell lines SHK-1 and TO despite using 1,000 and 2,000 times less virus, respectively. Rabbit antiserum to infectious pancreatic necrosis virus (IPNV) neutralized IPNV to the same degree in both CHSE-214 and TO cells. The poor neutralization of ISAV in TO cells was attributed to a possible Fc receptor mediated virus infectivity because: (1) neutralization by rabbit antiserum to ISAV was increased 48-fold in the presence of staphylococcal Protein A, and (2) using FITC-labeled virus and spectrofluorometry, a significant increase (p-value = 0.018) in fluorescence intensity of intracellular virus could be observed in assays of virus-antiserum mixtures in absence of Protein A compared to assays in presence of Protein A. Neutralization of ISAV with fish antisera was observed only in CHSE-214 cells as Protein A could not restore the neutralization on TO cells. In this study a panel of ISAV specific monoclonal antibodies (mAbs) was also characterized and used to see if it either neutralized

or enhanced virus replication in macrophage-like fish cell lines. High concentrations of mAbs 2D3 and 4A11, representing IgM and IgG isotypes respectively, neutralized ISAV strain NBISA01in TO cells. Lower concentrations of these mAbs did not enhance the viral infection in TO cells.

In conclusion this study demonstrated for the first time that (a) the mechanism of cell death during ISAV infection may be dependent on the cell type, (b) apoptosis in ISAV-infected cells is cell type specific, (c) TO cells undergo necrosis during ISAV infection, (c) apoptosis in ISAV-infected cells is caspase-dependent, and that (d) ISAV entry and replication in macrophage-like fish cell lines can be facilitated by virus-specific antibodies. These findings may have implications for the *in-vivo* pathogenesis and persistence of ISAV since two of the cell lines (SHK-1 and TO) used in this study were derived from the target cells of the virus in Atlantic salmon.

ACKNOWLEDGMENTS

This research project was supported by the Science and Engineering Research Canada (NSERC) Discovery and Strategic Grants to Dr. Frederick S.B Kibenge.

I thank Department of Pathology and Microbiology for the graduate student stipend and Atlantic Veterinary College for providing D.V.M graduate program award for my personal support.

A sincere appreciation goes to my supervisor Dr. Frederick S.B Kibenge for his excellent guidance and support throughout my Ph.D thesis research. I also extend my appreciation to the members of my supervisory committee, Dr. Basil Ikede (Chairman), Dr. Arnost Cepica, Dr. Dave Groman, Dr. Larry Hammell, and Dr. Laura Brown for their creative suggestions and critical reading of my thesis.

I would like to express my gratitude to Dr. Harry Hariharan and members of his family for all the help and support that I have received throughout my graduate program.

I thank Dr. Molly Kibenge for timely help and useful advice. I wish to thank Patricia McKenna and Dr. Biao Qian for excellent technical support.

I am grateful to the members of the faculty and staff of the Department of Pathology and Microbiology and fellow graduate students for their support and assistance.

A special gratitude goes to my father late Joseph Lucka Palliodil who provided me the inspiration and confidence and all of my family members in India for their prayers and encouragement.

Finally, I thank my wife Beena, son Joe, and daughter Mary Ann for their cooperation, understanding and continued support at all times.

Above all, I thank God almighty for providing everything to complete my program in the best way possible.

DEDICATION

Beena, Joe, and Mary Ann

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Abbreviation	Term
ABTS	2,2'-azino-di-[3-ethylbezthiazoline-6-sulfonic acid]
ADCC	antibody dependent cell mediated cytotoxicity
ADE	antibody-dependent enhancement
AIF	apoptosis inducing factor
AP	alkaline phosphatase
Bcl-2	B cell lymphoma-2
BSA	bovine serum albumin
CAD	caspase-activated deoxyribonuclease
CPE	cytopathic effect
DD	death domain
DGGE	denaturing gradient gel electrophoresis
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
dsRNA	double stranded RNA
DR	death receptor
DEVD	aspartate-glutamate-valine-aspartate

EDTA	ethylenediaminetetraacetate
eIF4E	eucaryotic initiation factor 4E
eIF4G	eucaryotic initiation factor 4G
eIF2	eucaryotic initiation factor 2
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
EST	expressed sequence tag
FADD	Fas-associated DD
FBS	foetal bovine serum
FcR	Fc receptors
FIP	feline infectious peritonitis
FITC	fluoresceine isothiocyanate
GDP	guanosine diphosphate
GFP	green fluorescent protein
gp	glycoprotein
Gpx	glutathione peroxidase
GTP	guanosine triphosphate
GRSF-1	G-rich sequence factor-1
HA	haemagglutinin
HE	haemagglutinin esterase
HI	haemagglutination inhibition
HIV	human immunodeficiency virus

HMGB 1	high mobility group box protein 1
HPR	highly polymorphic region
HRPO	horseradish peroxidase
HSV-1	herpes simplex virus-1
IFAT	indirect fluorescent antibody test
IFN	interferon
IPNV	infectious pancreatic necrosis virus
ISA	infectious salmon anaemia
ISAV	infectious salmon anaemia virus
K+	potassium ion
kDa	kilo Dalton
IAP	inhibitors of apoptosis protein
LPS	lipopolysaccharide
LMP-1	latent membrane protein-1
HMEM	minimum essential medium with Hank's salts
mAb	monoclonal antibody
MHC	major histocompatibility complex
mg	milligram
ml	millilitre
µm	micrometre
µg	microgram
MOCV	molluscum contagiosum virus

mRNA	messenger RNA
Na+	sodium ion
NA	neuraminidase
NEP	nuclear export protein
NK	natural killer cell
nm	nanometre
NP	nucleoprotein
NS	nonstructural
OD	optical density
OIE	Office International des Epizooties
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PKR	protein kinase RNA dependent
RNA	ribonucleic acid
RNase	ribonuclease
RNPs	ribonucleoproteins
vRNP	viral ribonucleoproteins
RRV	Ross river virus
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFM	serum free medium
SV40	simian virus 40
TBS	tris buffered saline
TTBS	tris buffered saline with 0.05% Tween 20
$TCID_{50}$	median tissue culture infectious dose
TdT	terminal deoxynucleotidyl transferase
TGF-β	transforming growth factor β
TNE	tris sodium chloride EDTA
TNF	tumour necrosis factor
T-PBS	phosphate buffered saline with 0.05% (v/v)Tween 20
TRAIL	TNF-related apoptosis-inducing ligand
rRNA	ribosomal RNA
tRNA	transfer RNA
TFIID	transcription factor IID
TUNEL	terminal dUTP Nick End-Labeling assay
UTR	untranslated region
UV	ultraviolet
VN	virus neutralization
VSV	vesicular stomatitis virus

Chapter 1

General introduction and literature review*

* Portions of this chapter have been published in:

Kibenge, F.S.B., Munir, K., Kibenge, M.J.T., **Joseph, T.**, Moneke. E. 2004. Infectious salmon anaemia virus: the causative agent, pathogenesis, and immunity. *Animal Health Research Reviews. In press.*

1.1. General introduction

Infectious salmon anaemia virus (ISAV) is the causative agent of infectious salmon anaemia (ISA) which is an economically important disease of farmed salmonid fish in the Northern hemisphere. This virus has been classified in the family *Orthomyxoviridae* (Krossøy et al., 1999), genus *Isavirus* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). The virus is considered to be such a threat to the fish farming industry that the European Union listed ISA as the most dangerous of fish diseases. ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon (Nylund et al., 1994, 1996; Dannevig et al., 1995a,b; Byrne et al., 1998; Rodger et al., 1998) and asymptomatic infection in feral fish (Raynard et al., 2001a), a situation analogous to avian influenza viruses in domestic poultry and feral birds (Kibenge et al., 2004). The immune response following ISAV infection does not provide full protection against the disease; high mortality has usually been observed in both naturally and experimentally infected fish (Thorud 1991; Dannevig et al., 1993; Falk et al., 1995). Atlantic salmon that recovered from a clinical infection can continue to shed virus for long periods of time. Detection of ISAV in carrier fish required the use of RT-PCR (Devold et al., 2000) suggesting that the viral RNA is not abundant. There is lack of adequate information on the host-pathogen interactions of this virus. Such information is essential to understand the mechanisms of pathogenesis and persistence of ISAV.

In Atlantic salmon, ISAV targets leucocytic cells (Falk et al., 1995) and endothelial cells (Falk & Dannevig 1995) or endothelial-associated cells (Falk et al., 2001), and macrophages (Moneke et al., 2003). *In-vitro* ISAV readily replicates in salmon head kidney (SHK-1), TO,

and Atlantic salmon kidney (ASK-2) cells which are macrophage-like cell lines derived from the Atlantic salmon pronephros (Dannevig et al., 1995; Wergeland & Jakobsen 2001; Rolland et al., 2002). Some strains of ISAV can also replicate and cause CPE in the Chinook salmon embryo (CHSE -214) cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001). These cell lines differ in their growth characteristics (media requirements, split ratio, cell composition), and they display distinctly different viral-induced CPE (Kibenge et al., 2001; Rolland et al., 2002).

Generally, morphological changes referred to as CPE result from virus-cell interactions. Cytopathic effects include cell rounding, cell lysis, cell fusion to form syncytia, or more subtle changes in cell shape. Many pathophysiological changes occur in cells infected with cytopathic viruses and death of the cell may be the final result of the cumulative action of many insults. In general, the mechanism of cell death during virus infection involves both necrosis and programmed cell death or apoptosis (Hardwick and Griffin 1997). Conversely induction of apoptosis may also represent a mechanism by which virus infection is limited. Viruses have evolved various strategies to inhibit and/or promote apoptosis at different stages in their replicative cycles in host cells. The mechanism of cell death during ISAV infection and the role of apoptosis in ISAV pathogenesis have not been studied previously.

The ability of a virus to infect and replicate within the cells of mononuclear phagocyte system can be a major factor in the pathogenesis of a virus infection because mononuclear phagocytes can facilitate access of viruses to susceptible tissues and organs, thereby

hastening the infection process. The paradoxical ability of antiviral antibody to enhance virus infection of Fc receptor-bearing cells of mononuclear phagocyte system has been demonstrated across a wide range of virus families (Halstead and O'Rourke 1977; Sullivan 2001; Tirado and Yoon 2003). In the case of ISAV, no information is available on how the antibody-virus complexes interact with the host macrophages.

In this thesis research two important aspects of host-pathogen interactions, (i) the mechanism of cell death during ISAV infection as well as, (ii) antibody-mediated growth of ISAV in macrophage-like fish cell lines, were investigated. This work is presented as follows: Chapter 1 reviews the literature on virus-host cell interactions, virus neutralization, antibody dependent enhancement of viral infection, and the pathogenesis and immunology of ISAV. General materials and methods are described in Chapter 2. Studies on mechanisms of cell death during ISAV infection are presented in Chapter 3. In Chapter 4, studies on antibody-mediated growth of ISAV in macrophage-like fish cell lines are described. The characterisation of ISAV-specific mAbs including their analysis for enhancement of ISAV infection in macrophage-like fish cell lines are presented in Chapter 5. Chapter 6 is then a general discussion of the results, conclusions, and directions for future studies.

1.2. Literature review

1.2.1. Virus-host cell interactions

Viruses are ubiquitous and often dangerous obligate intracellular parasites that exploit the cellular metabolism for self propagation. During replication viruses interfere with normal

cellular functions in ways harmful to the host cell. Many viruses inhibit host gene expression and have evolved various strategies to shut down the synthesis of cellular macromolecules and direct the cellular machinery to produce viral components (Lyles 2000). Studies on latent and persistent viruses in particular have shown that viruses have developed extremely sophisticated strategies to manipulate the host's cellular regulation for successful replication or persistence (Krajsci and Wold 1998). The mechanisms of cell death during virus infection involve both necrosis and apoptosis. Apoptosis can result from virus-induced inhibition of host gene expression or antiviral host response (Hardwick and Griffin 1997). It is important to study virus-cell interactions because the mechanisms of cell death and factors that influence the balance of viral cytopathogenesis and the host antiviral response apparent in cell cultures are likely to be major determinants of the ability of viruses to cause disease in animal hosts (Lyles 2000).

1.2.1.1. Viral effect on host protein synthesis

1.2.1.1.1. Inhibition of host transcription

Inhibition of host protein synthesis is a major feature of virus-infected cells and it can result from either a direct effect of the viral gene products or activation of host responses to viral infection. DNA transcription is the process by which all types of RNA (mRNA, tRNA, and rRNA) are copied from a DNA template by RNA polymerase enzymes. Various transcription factors - DNA-binding proteins - interact with promoters and are necessary for initiation of RNA synthesis. Host transcriptional machinery is one of the main targets of most RNA viruses, since these viruses, with the exception of orthomyxoviruses replicate in the

cytoplasm of the host cell without any requirement for host transcriptional activity. For many RNA viruses such as picornaviruses and rhabdoviruses, the inhibition of host RNA synthesis is a prominent feature of virus infection (Lyles 2000). Proteins encoded by poliovirus (picornavirus) and vesicular stomatitis virus (rhabdovirus) inhibit transcription by all three host RNA polymerases (Fernades-Tomas 1982; Lyles et al., 1988). The 3C protease of poliovirus and M protein of vesicular stomatitis virus are the viral gene products responsible for inhibiting host transcription (Ferran et al., 1997; Yalamanchili et al., 1997). Transcription factor TFIID isolated from poliovirus or vesicular stomatitis virus-infected cells was not able to initiate transcription in the presence of other transcription factors and RNA polymerase II from uninfected cells (Yalamanchili et al., 1997; Yuan et al., 1998). Therefore, the mechanism of transcription-inhibition may involve cleavage and inactivation of transcription factors that play a central role in host cell transcription.

1.2.1.1.2. Inhibition of processing and transport of host RNA

The pre-messenger RNA (pre-mRNA) produced by transcription is processed mainly by 5'-capping (addition of a 7-methylguanosine nucleotide to the 5' end), polyadenylation (addition of poly(A) tail to generate the mature 3' end), and splicing (removal of noncoding - intron - sequences) before leaving the nucleus for translation in the cytoplasm. Virus encoded proteins can interfere with various levels of posttranscriptional processing and transport of host RNA to prevent an antiviral host response since many processes required for the expression of host genes are not required for the expression of viral genes. In the case of Influenza A virus infection, a single viral protein, NS1 protein, inhibits at least two steps in

the processing of the 3' ends of host mRNA and also inhibits the splicing of host mRNA (Fortes et al., 1994; Qiu et al., 1995; Nemeroff et al., 1998; Chen et al., 1999). Different protein domains of NS1 protein are responsible for different activities (Qian et al., 1994, 1995). NS1 protein binds poly(A) of mRNA both directly and indirectly and inhibits the polyadenylation of pre-mRNA and retains cellular RNA polymerase II transcripts in the nucleus (Chen et al., 1999). Biochemical analyses have shown that in the presence of NS1 protein pre-mRNA assemble into spliceosomes that are unable to catalyze the two steps of the splicing reaction, thus inhibiting the splicing of host mRNAs (Lu et al., 1994). Based on *in-vitro* studies it has been suggested that the NS1 protein may inhibit pre-mRNA splicing by binding to U6 RNA within the spliceosome (Qian et al., 1995). Inhibition of polyadenylation and splicing would result in an increase in the intranuclear concentration of the capped cellular mRNAs that could be used by the viral polymerase for priming transcription. Post-transcriptional control of gene expression can also occur at the level of nucleocytoplasmic transport of a mature, spliced mRNA. NS1 protein of Influenza A virus and M protein of vesicular stomatitis virus are known to inhibit nuclear-cytoplasmic transport of the host mRNA and thus interfere with protein synthesis in the host cell (Alonso-Caplen et al., 1992; Her et al., 1997).

1.2.1.1.3. Inhibition of translation of cellular proteins

The complex process of translating mRNA into proteins involves three major steps, (i) initiation (the assembly of a ribosome on an mRNA), (ii) elongation (repeated cycles of amino acid delivery, peptide bond formation and movement of ribosomes along the mRNA),

and (iii) termination (the release of the polypeptide chain). Many viruses employ multiple mechanisms to control translation of cellular mRNAs. Mechanisms of translation-inhibition include inactivation of translation factors, degradation of host cell mRNAs, production of factors that specifically inhibit cellular mRNA translation or facilitate viral mRNA translation, viral mRNA that outcompete cellular mRNAs for the translational machinery, and changes in the intracellular ionic environment that favour translation of viral mRNA (Hardwick and Griffin 1997). In many cases, the inhibition of protein synthesis may also be due to the antiviral response of the host. Cleavage of translation initiation factor eIF4G by viral 2A protease in picornavirus-infected cells is a classic example of host translation-inhibition by inactivation of translation factors. Cleavage of eIF4G inhibits translation of capped but not uncapped mRNAs. Since picornaviruses lack a cap structure, this inhibition does not affect the translation of viral mRNAs (Porter 1993). Picornaviruses also alter the intracellular ionic environment by increasing the concentration of Na+ and decreasing that of K+. Increased Na+ concentration decreases the cellular mRNA translation and improves the translation of viral mRNAs (Carrasco et al., 1977). Inhibition of cellular protein synthesis by degradation of cellular mRNAs has been observed during coronavirus infection (Kyuwa et al., 1994).

Inhibition of cellular protein synthesis due to the antiviral host response occurs during vesicular stomatitis virus infection. This is mainly due to the response to dsRNA which is a byproduct of replication of nearly all viruses. A major feature of the response of host cells to viral dsRNA is the inhibition of protein synthesis by RNA dependent protein kinase (PKR)

(Hardwick and Griffin 1997; Lyles 2000). While PKR is known primarily as an interferon-inducible kinase, most cells constitutively express substantial amounts, and it serves as an important activator of the host response to dsRNA even in the absence of interferon. In the presence of dsRNA, PKR phosphorylates the alpha subunit of the GTP-binding translation initiation factor eIF2. Phosphorylation of the alpha subunit locks eIF2 in the GDP-bound form, preventing its reutilization for translation initiation (Hardwick and Griffin 1997). Vesicular stomatitis virus is an example of a virus which was shown to induce inhibition of eIF2 by preventing its reutilization, a hall mark of PKR activity (Centrella et al., 1982; Dratewka-Kos et al., 1984). The cap binding translation initiation factor eIF4E is the target of inactivation in Influenza A virus infected cells. In Influenza A virus-infected cells, eIF4E is inactivated by dephosphorylation which reduces its ability to bind to capped cellular mRNAs to initiate translation (Feigenblum and Schneider 1993). Influenza virus is able to overcome the inhibition of host cell mRNA transport and translation by establishing its own translational and transport regulatory mechanisms. Studies have shown that a cellular protein G-rich sequence factor-1 (GRSF-1) which specifically binds to the 5' untranslated region (UTR) of viral mRNAs but not to the cellular mRNAs, which may ensure the selective translation of viral mRNAs in infected cells (Park et al., 1999). It is not yet known however, how GRSF-1 affects the overall translation efficiency of influenza virus mRNAs.

In order to maintain the cell as competent for the preferential translation of viral mRNAs, Influenza A virus has developed two means of escape from the inhibitory effects of PKR. First, the NS1 protein inhibits the interferon response mediated by PKR by binding to

dsRNA as well as the PKR enzyme, leading to blockage of PKR activation (Lu et al., 1995). Second, Influenza A virus infection activates a latent chaperon-associated protein, p58IPK, which can interfere with PKR dimerization and activation (Tan et al., 1998). Some of the viral proteins can interfere with the cellular protein synthesis by inhibiting the transport of the cellular proteins from endoplasmic reticulum leading to the degradation of those proteins. Adenovirus E3/19K protein binds and retains the heavy chain of the major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (Paabo et al., 1989).

Virus-cell interaction can also lead to the alteration of plasma membrane permeability, induction of synthesis of new intracellular membranes by stimulating the synthesis of phospholipids, and induction of rearrangement of previously existing membrane. Enveloped viruses insert viral proteins into the plasma membrane which may lead to cellular membrane fusion and syncytia formation (Hardwick and Griffin 1997).

1.2.1.2. Virus induced cell death: apoptosis versus necrosis

Classically, virologists have assumed that viruses kill cells by taking over their transcriptional and translational machinery and disrupting their membrane integrity. That is, the infected cell is disrupted to such a degree that it cannot maintain viability and the cell dies by necrosis. Some viruses are capable of such lethal parasitism whereas other viruses kill cells by triggering apoptosis (Hardwick and Griffin 1997). Necrosis affects massive and contiguous cells with the affected cells showing irregular clumping of chromatin, pyknosis or karyolysis, random cleavage of the DNA, collapse of nuclear membrane, very early

swelling of cell and intracellular organelles, and loss of integrity and membrane collapse ultimately leading to cell lysis. Rupture of the cell causes release of its contents into the surrounding environment which will lead to extensive inflammatory reaction (Cotran et al., 1999). Recent studies have shown that release of a high mobility group 1 (HMGB 1) protein, a chromatin binding factor, from necrotic cells but not from cells undergoing apoptosis, triggers the inflammatory reactions caused by necrosis (Scaffidi et al., 2002). In contrast, apoptosis is an energy-dependent cell death that displays distinct hall marks, including cytoplasmic shrinkage, chromatin condensation, intranucleosomal cleavage, phosphatidylserine exposure, plasma membrane blebbing, activation of caspases, and cell fragmentation into apoptotic bodies which are phagocytosed without provoking an inflammatory response (Cotran et al., 1999; Schultz and Harrington 2003).

Apoptosis was initially recognized in 1972 by its distinct morphology and named after the Greek designation for "falling off". It is a form of cell death designed to eliminate unwanted host cells through activation of a genetically programmed and internally controlled self-destructive pathway. It occurs during development, as a homeostatic mechanism to maintain cell populations in tissues, or as a defence mechanism such as in immune reactions, when cells are damaged by disease or noxious agents, and in aging (Cotran et al., 1999). One of the important functions of apoptosis is to eliminate cells infected with viruses in order to prevent the replication and spread of the virus and progression of disease (Hay and Kannourakis 2002). Virus survival is dependent upon effective exploitation of the existing cellular machinery. Therefore, viruses have developed various strategies to affect the

apoptotic process within a host cell. Viruses may instigate either promotion or inhibition of apoptosis, but often they are harmed by the natural apoptotic action elicited by the host cell. Virus-induced apoptosis may also be harmful to the host in cases when the virus infects a cell population that cannot be replenished, for example, Sindbis virus induced apoptosis of neurons (Lewis et al., 1996; Hardwick and Griffin 1997). Also, a virus can establish a persistent infection by inhibiting apoptosis, contributing to the pathogenesis of the disease (Hay and Kannourakis 2002). Although the benefits to a virus in avoiding the apoptotic process are obvious, the onset of apoptosis is in some cases also advantageous. The apoptotic bodies formed during apoptosis are consumed by the phagocytic action of the neighbouring cells. This engulfment provides a means for the dissemination of the virus without initiating a concomitant host response, which would allow the release of the progeny virus into the extracellular fluid. This was shown in a recent study involving an adenovirus vector that proliferated in human cancer cells (Mi et al., 2001).

1.2.1.2.1. Apoptotic signalling pathways

Two major signalling pathways of apoptosis are the extrinsic pathway and the intrinsic pathway (Schultz and Harrington 2003). The extrinsic pathway is activated when a specific protein ligand binds to a cell surface transmembrane receptor. Apoptotic signals are transmitted to target cells via the TNF superfamily of death receptors (DRs), the members characterized by a conserved extracellular cysteine-rich motif. Six different DRs are known: Fas, TNFR1, DR3, TNF-related apoptosis-inducing ligand (TRAIL) (TRAIL-R1 or DR4, and TRAIL-R2 or DR5), and DR6 (Schultz and Harrington 2003). One of the best-characterized

surface receptors is Fas, a 319-amino acid type 1 transmembrane glycoprotein, with broad distribution on both lymphoid and non-lymphoid cells (Itoh et al., 1991). An apoptotic signal is initiated in target cells when Fas is engaged by its natural ligand FasL or by anti-Fas antibodies. The interaction may occur on the effector cell, or in some cases, on the target cell. FasL, a trimeric type 2 transmembrane protein, and its binding to Fas causes receptor oligomerization (Wallach et al., 1999). The death domain (DD) of the receptor then recruits adaptor proteins such as Fas-associated DD (FADD) protein (Muzio et al., 1996). Adapter proteins have a DD at its C-terminus and a second protein-protein interaction domain, called a death-effector domain, at its N-terminus (Wallach et al., 1999). The death effector domain of the adaptor protein binds to the death-effector domain, or prodomain, of caspase-8, and a complex termed the death-inducing signalling complex (DISC) is formed which subsequently signals proteolysis and endonucleolytic cleavage in target cells. Therefore, FADD recruits procaspase-8 to the Fas signaling complex to form DISC (Muzio et al., 1996; Wallach et al., 1999). Binding of TNF-α with TNFR1 also leads to the formation of DISC via its association with TNFR1-associated DD, and FADD. The overall result after recruitment to the DISC is the autocatalytic processing that leads to activation of caspase-8 (Wallach et al., 1999).

Caspases are aspartyl-specific cysteine proteases and they are essential for execution of apoptosis. They are synthesized as proenzymes that are activated by proteolytic cleavage. There are 14 known mammalian caspase-family members, and they form an intracellular proteolytic cascade that modulates many cellular events in the apoptotic pathway, including

activation of transcription factors and cleavage of the structural proteins that lead to characteristic morphological changes, e.g., membrane blebbing, and apoptotic body formation during apoptosis (Degterev et al., 2003). In general, activation of upstream caspases or initiator caspases such as caspase-8 and 9, lead to the activation of downstream effector caspases such as caspase-3, 6, or 7. Caspase-3 activates its target, the caspase-activated deoxyribonuclease (CAD), an endogenous endonuclease. CAD exists as an inactive complex until its inhibition is cleaved by activated caspases, thus releasing CAD (Sakahira et al., 1998). The latter enters the nucleus and degrades the chromosomal DNA. The cellular DNA is cleaved between nucleosomes, giving DNA fragments of multiples of 180-200 bp. The apoptotic DNA fragments can be visualized by agarose gel electrophoresis as "DNA ladder" which is a hall mark of apoptosis (Sakahira et al., 1998; Schultz and Harrington 2003).

There is also evidence suggesting that not all apoptotic cell death requires caspase activation. Adenovirus E4orf4 protein has been shown to induce caspase-independent apoptosis in Chinese hamster ovary (CHO) cells (Lavoie et al., 1998). TNFα, an inducer of classic caspase-dependent apoptosis, can also induce non-classical caspase-independent apoptosis (Maianski et al., 2003). Most recently, a study on caspase independent apoptosis showed that serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions (Egger et al., 2003). Currently, however, little is known about caspase-independent cell death, and its physiological relevance awaits further clarification.

The intrinsic pathway of apoptosis involves cellular organelles such as mitochondria. The involvement of the mitochondria in apoptosis first came to light when it was discovered that one of the critical factors required for the activation of caspase 3 was cytochrome c. Disruption of the membrane potential of mitochondria led to the release of cytochrome c into the cytosol where cytochrome c interacts with Apaf-1 and pro-caspase 9 to form a complex termed the apoptosome. Pro-caspase 9 is then autocatalytically processed to mature caspase and initiates a caspase cascade downstream in order to execute apoptosis (Li et al., 1997). Other mitochondrial proteins such as apoptosis-inducing factor (AIF) can also be released by mitochondria to promote apoptosis in a caspase-dependent or independent manner (Green and Reed 1998). Several proteins that reside in the inner and outer membranes of the mitochondria may form ion channels to regulate the efflux of cytochrome c (Luo et al., 1998). One such regulatory protein is Bcl-2. Bcl-2 is part of a family that can be differentiated into anti-apoptotic (e.g., Bcl-2, Bcl-x_L) and pro-apoptotic (e.g., Bax, Bad, Bak) proteins (Adams and Cory 1998). Bcl-2 itself is an integral membrane protein located mainly on the outer membrane of the mitochondria and it modulates the cytochrome c/Apaf-1/caspase-9 pathway of apoptosis by regulating the liberation of cytochrome c (Yang et al., 1997). The balance between these pro-apoptotic and anti-apoptotic factors controls the fate of the cell (Adams and Cory 1998).

1.2.1.2.2. Mechanism of induction of apoptosis by viruses

There are several mechanisms by which viruses activate the apoptotic pathway. Influenza virus infection induces the expression of Fas, and Fas-mediated apoptosis has been suggested

to be an important mechanism of cell death during influenza virus infection. The role of Fas in Influenza A virus-induced apoptosis is also supported by the activation of caspase-8 but not caspase-9 in virus-infected cells (Balachandran et al., 2000). It has been shown that the induction of Fas expression by influenza virus or viral dsRNA is mediated partly by PKR, indicating the involvement of antiviral response of the host cell in the induction of apoptosis during Influenza A virus infection (Balachandran et al., 2000). Caspase-mediated cleavage of NP protein of human strains of Influenza virus A also supports the antiviral role of apoptosis during influenza virus infection (Zhirnov et al., 1999). In this case, caspase activation may limit virus production as the truncated form of NP cannot be used for proper virus assembly (Zhirnov et al., 1999).

The role of various viral proteins during influenza virus-induced apoptosis has also been studied. It has been shown that NS1 protein itself is capable of inducing apoptosis when expressed in cell cultures (Schultz-Cherry et al., 2001). Other studies however suggest that NS1 possesses anti-apoptotic potential as well (Zhirnov et al., 2002b). Experiments in Madin Darby canine kidney (MDCK) cells demonstrated that NA could activate latent transforming growth factor β (TGF-β) to its biologically active form, a broad inducer of apoptosis (Morris et al., 1999). Inhibitors of NA delayed the onset of apoptosis when added shortly after infection. Also, viruses with highly active NA induced apoptosis in host cells more rapidly than did those with less active NA (Morris et al., 1999). Interaction of M1 protein of Influenza A virus with cellular caspase-8 suggest that M1 protein may have a role in virus-induced apoptosis (Zhirnov et al., 2002a). A new influenza virus gene product, PB-F2,

produced by a +1 reading frame in the viral RNA segment which encodes polymerase subunit PB1 has also been shown to play important roles in influenza virus-induced apoptosis (Chen et al., 2001). Thus, influenza viruses may have multiple mechanisms that contribute to the induction of apoptosis in host cells.

Earlier studies also suggested that apoptosis facilitated replication of Influenza virus in cell cultures. Overexpression of anti-apoptotic protein Bcl-2 inhibited Influenza A virus-induced apoptosis as well as reduced virus production in MDCK cells, and the blocking effect was attributed to modified glycosylation of HA protein (Olsen et al., 1996). This argument is supported by a recent study which demonstrated that caspase-3 activation is essential for Influenza A virus propagation in cell cultures (Wurzer et al., 2003). Inhibition of caspase-3 activity by caspase-3 inhibitor or small interfering RNAs blocked efficient replication of Influenza A virus in cell cultures. Inhibition of virus replication correlated with the retention of viral RNP complexes in the nucleus, which prevented production of progeny virus particles (Wurzer et al., 2003).

Some viruses may induce apoptosis through the direct action of specific viral proteins. A good example is adenovirus early protein 1A (E1A). E1A induces cell death by a tumour suppressor protein p53-dependent mechanism. The tumour suppressor protein p53 is a transcriptional regulator that can also induce apoptosis in response to virus infection as well as in response to many other stimuli such as DNA damage or unscheduled DNA synthesis. E1A protein is also found to sensitize cells to apoptosis by TNF and FasL by unknown

mechanisms (Lowe et al., 1993; Roulston et al., 1999). Other viruses induce apoptosis indirectly through their effects on cellular functions. Poliovirus infection activates the apoptotic pathway, involving mitochondrial damage, cytochrome c efflux, and consecutive activation of caspase-9 and caspase-3 (Belov et al., 2003). In some disease states, apoptosis occurs only in those cells infected with the virus, for example, Sindbis virus infection of neurons (Lewis et al., 1996). It is also conceivable that a virus-infected cell could cause apoptosis in an adjacent uninfected cell by a number of mechanisms, including secretion of factors that activate the death program. A good example is the apoptosis of CD4+ T cells in HIV infection (Ameisen and Capron 1991).

Recent studies have demonstrated the existence of a novel pathway in which caspase-12 functions as the initiator caspase in response to toxic insult to endoplasmic reticulum (ER). Endoplasmic reticulum provides a unique compartment for the post-translational modification, folding, and oligomerization of newly synthesized proteins. The ER is also considered to be the major signal-transducing organelle. Several endogenous imbalances in the cell often contribute to the malfunction of the ER (also called ER stress), including massive protein production, loss of Ca++ homeostasis, inhibition of N-linked glycosylation, and accumulation of mutant proteins (Pahl 1999). Caspase-12 is specifically activated in cells subjected to ER stress. Caspase-12 is associated with the cytoplasmic face of the ER and cleaved into an active form by caspase-7 in response to prolonged ER stress. The mechanism of caspase-12 mediated apoptosis has however not been elucidated. It has been suggested that caspase-12 may activate caspase-3 directly or through the activation of caspase-9 or caspase-

12 might induce apoptosis directly (Rao et al., 2001). Viruses that utilize the host cell ER as an integral part of their life cycle would be predicted to cause some level of ER stress (eg. viruses of *Flaviviridae* family). It has been shown that Japanese encephalitis virus and cytopathic strains of bovine viral diarrhoea virus induce ER stress leading to caspase-12 activity and ER-stress induced apoptosis (Jordan et al., 2002; Su et al., 2002).

1.2.1.2.3. Viral inhibition of apoptosis

Apoptosis can also be a protective host response to eliminate virus- infected cells. Therefore, viruses use various strategies to inhibit apoptosis or to complete their replication cycles before the cell expires. A virus may multiply rapidly to produce many progeny virions before an effective host response can be mounted. This approach is exhibited by most RNA viruses, including Influenza virus (Kurokawa et al., 1999). Another strategy is to remain cryptic such that a virus may infect a cell and remain undetected, thus avoiding host cell destruction and allowing a productive infection (Paroli et al., 2000). Viruses influence the extrinsic pathway of apoptosis from the point of initiation. Adenovirus proteins E3-10.4K and E3-14.5K reduce the presentation of Fas molecules on the cell surface leading to a resistance to Fas-mediated cell death. These adenoviral proteins also provide protection against TNF-mediated cell death by an unknown mechanism (Dimitrov et al., 1997; Shisler et al., 1997). Epstein-Barr virus produces an anti-apoptotic protein namely latent membrane protein-1 (LMP-1). This protein accumulates in the host cell plasma membrane and interferes with TNF-mediated apoptosis (Eliopoulos et al., 1999). The myxoma virus protein M-T2 is a mimic protein of the TNF receptor and is a potent inhibitor of extracellular TNF-mediated apoptosis. It binds

TNF, thus preventing TNF receptor signal transduction (Xu et al., 2000). A few virus caspase inhibitors have been identified as well, including cowpox virus CrmA, baculovirus IAP (inhibitors of apoptosis protein) and p35, and African swine fever virus product A224L/4CL. CrmA is a potent caspase-1/casapse-8 inhibitor. Baculovirus protein p35 is a wide-ranging caspase inhibitor, inhibiting mammalian caspase-1- 4 and 7. Baculovirus-induced IAP inhibits caspase-3, 6, and 7. The A224L gene produces a viral IAP related-protein and this protein has been shown to interact with, and suppress, the activity of caspase-3 (Deveraux et al., 1997).

Many viruses interfere with the intrinsic pathway (mitochondria-mediated) of apoptosis by encoding proteins that mimic Bcl-2 family proteins. The E1B-19K protein of adenovirus is an example of such a mimic. It has been shown to be associated with and inhibit apoptosis induced by the pro-apoptotic Bcl-2 protein family members (Han et al., 1996). Epstein-Barr virus proteins BHRF1 and BALF1 also show similarity to anti-apoptotic Bcl-2 family proteins, both in structure and function and inhibit mitochondria-mediated apoptosis (Marshall et al., 1999). Marek's disease virus apparently controls the transcription of apoptosis-related genes by the induction of Bcl-2 and by the repression of Bax (Barry and McFadden 1998).

For many viruses, replication depends on the induction of S phase, often leading to increased levels of tumour suppressor protein p53. Activated p53 leads to apoptosis. Therefore viruses have devised strategies for countering p53 to maximize progeny production (Roulston et al.,

1999). Adenovirus E1B protein and the large T antigen of SV40 (simian virus 40) bind to p53 and inhibit p53-mediated apoptosis (Jung and Yuan 1997). Similarly herpesviruses, retroviruses, and flaviviruses have also evolved genes to inhibit p53-mediated apoptosis (Friborg et al., 1999; Hay and Kannuorakis 2002).

Oxidative stress is a well-established feature of apoptosis. The production of reactive oxygen intermediates and the accumulation of oxidized cellular compounds play a role in the cell death mediation. Glutathione peroxidase (GPx) manages the deleterious effects of these stressing agents in the cell (Buttke and Sandstorm 1994). It has been shown that molluscum contagiosum virus (MOCV) is able to encode a protein similar to Glutathione peroxidase which apparently confers survival advantage by inhibiting apoptosis (Shisler et al., 1998).

1.2.1.2.4. The interferons and apoptosis

A host response to virus infection includes the induction of interferons (IFN). The IFN were discovered in the late 1950s by their capacity to inhibit influenza virus replication. There are two types of interferons. Type I IFN (α and β) are induced by most cell-types such as leukocytes and fibroblasts. Type-II IFN (γ) is mainly secreted by Th-1 lymphocytes and natural killer (NK) cells. A third member (referred to as ω) has also been described (Barber 2001). Although it is clear that IFN can prevent the replication of many viruses such as vesicular stomatitis virus (VSV) without damaging the host cell, evidence also indicates that these cytokines can greatly sensitize certain cell lines to apoptosis (Stark et al., 1998). Therefore, IFN plays an important role in the host response to viral infection by inducing

important cellular genes, such as PKR, 2'5'-oligoadenylate synthetase, and Mx genes (Stark et al., 1998). It has been shown that induction of PKR can sensitize the cell for apoptosis. The mechanism by which IFN appears to sensitize cells to apoptosis appears to be predominantly via the FADD/caspase-8 signalling, since IFN-induced cell death can be prevented by inhibitors of caspase-8. IFN-induced proteins (e.g. PKR) may govern the regulation of death-induced signalling complexes (DISCs) that comprise FADD/caspase-8 (Barber 2001). RNaseL, another IFN-induced enzyme, also induces apoptosis in virus-infected cells (Diaz-Guerra et al., 1997).

Several viruses have developed mechanisms to protect against the apoptotic and antiviral effects of interferon. The poxvirus Vaccinia encodes at least two proteins (E3L and K3L) that inhibit PKR action. E3L is a dsRNA binding protein of Vaccinia virus that prevents the interaction of PKR with viral RNA. E3L can inhibit apoptosis induced by infection with E3L mutant virus, or by over-expression of activated PKR or by over-expression of RNaseL and 2'5' oligoadenlylate synthetase (Davies et al., 1993). Inhibitors from other viruses include Influenza A virus NS1, which binds to PKR and prevents phosphorylation and inactivation of eIF-2α (major substrate of PKR) and herpesvirus (HSV-1) protein γ_1 34.5 that inhibits PKR-induced inhibition of protein synthesis and apoptosis (Roulston et al., 1999).

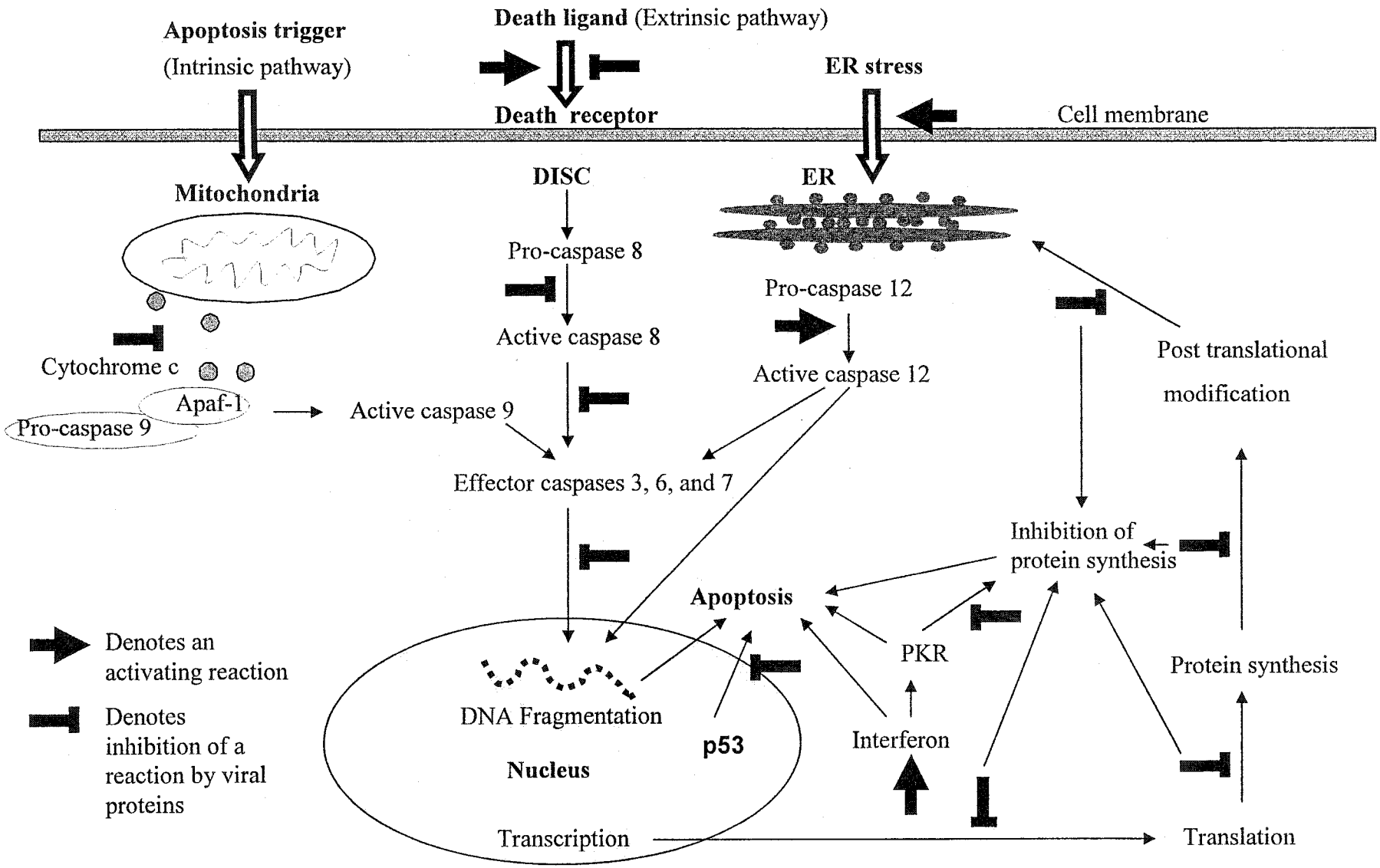


Fig 1.1. The relationship between apoptotic signaling pathways, host protein synthesis, and viral proteins (modified from Hay and Kannourakis 2002)

1.2.2.Virus neutralization

Virus neutralization tests have been valuable for obtaining knowledge of the antigenic properties of a virus. This is useful in understanding the immune response to viruses and designing vaccination strategies. Neutralization of viruses is defined as the abrogation of virus infectivity by the binding of antibodies to a virus particle (Dimmock 1995; Klasse and Sattenau 2002). The term neutralization is best used only in relation to *in-vitro* systems as there is little information about how antibodies act *in-vivo* (Dimmock 1995). As a minimum requirement for neutralization, the paratopes (antigen-binding sites) of antibodies must interact with epitopes on virion-associated antigens. The epitopes must be exposed on the surface of the virion, although exposure may be only transient or contingent upon interactions with receptors or other antibodies (Kjellen and Pereira 1968, Roivainen et al., 1993, Thali et al., 1993, Li et al., 1994). Binding of antibody to an epitope is not in itself sufficient for neutralization, and neutralizing epitopes must have the unique functional property which leads to loss of virus infectivity (Dimmock 1995). Neutralizing antibodies can be IgG, IgA or IgM. Data are lacking on the neutralizing functions of IgD and IgE. The relative neutralizing efficiency of different isotypes is not known.

1.2.2.1. Mechanisms of virus neutralization

The mechanism of neutralization may vary depending on the isotype and number of antibody molecules bound per virus particle. It has been shown that at low concentration, IgA antibodies did not prevent the attachment of influenza virus to the host cell receptors but neutralized the virus by inhibiting virus fusion activity. At high IgA concentration,

neutralization resulted from the failure of virus to attach to the host cell, presumably because the length of antibody projecting did not allow virus to make contact with the cell receptor (Armstrong and Dimmock 1992). Studies have shown that the IgM antibodies, despite their large size, prevented fewer than 50% of the neutralized virus from attaching to cells at physiological temperatures. The neutralized virus was not however internalized, suggesting that IgM antibodies interfere with the endocytic event responsible for internalization of the virus, by some unknown mechanism (Taylor and Dimmock 1985). IgG antibodies neutralize Influenza virus primarily by inhibiting virus-cell fusion, although at higher concentrations virus attachment to target cells was also inhibited (Edwards and Dimmock 2000; Knossow et al., 2002). A virus can be neutralized by various ways and the mechanism of neutralization can be classified according to the event in the virus replication cycle blocked by the antibodies.

1.2.2.1.1. Aggregation

Aggregation is a mechanism of neutralization that reduces the number of cells to which a virion can attach. For example, when ten infectious units are aggregated by an antibody to form a single infectious unit then the infectivity is effectively reduced 10-fold. In this way even non-neutralizing antibodies become neutralizing (Dimmock 1995). The importance of aggregation as a mechanism of neutralization varies greatly between viruses. For example, aggregation with IgG, IgM or IgA appears to add little to intrinsic neutralization of Influenza A virus but can be very effective in neutralizing poliovirus (Thomas et al., 1986; Armstrong et al., 1990; Outlaw et al., 1990).

1.2.2.1.2. Interference with viral attachment

Virus attachment to the target cell is necessary for infection. Therefore, blocking viral attachment is an important mechanism of neutralization of many naked and enveloped viruses (Burton et al., 2001). The mechanism of neutralization by steric inhibition of contact between the viral attachment site and its cell receptor occurs only rarely, and a direct quantitative relationship has been established for only very few virus-IgG mAb combinations. Newcastle disease virus is neutralized in this way by some of its mAbs (Iorio et al., 1989). Neutralization by this mechanism is thought to be inefficient because the virus will not be neutralized until all the attachment sites are blocked which would require binding of several antibodies per virion (Smith et al., 1993). Since it will take longer for the virus to bind several antibody molecules than one molecule, this type of neutralization is intrinsically less efficient than that requiring only one antibody molecule per virion (Dimmock 1995). Neutralization epitopes on the influenza virus haemagglutinin surround the receptor-binding pocket. This suggests that neutralization interferes with receptor binding and that mutation at these sites may abrogate antibody binding but not the docking of the receptor into its pocket (Skehel and Wiley 2000). Certain viruses, including herpes simplex virus and foot-and-mouth disease virus, make use of ancillary receptors such as heparan sulphate proteoglycans for initial tethering to the target cells, and they rely on other molecules for internalization or triggering the fusion (WuDunn and Spear 1989, Fry et al., 1999, Ugolini et al, 1999). These interactions involving various molecules are potential targets for neutralizing antibodies. In the case of poliovirus and HIV-1, it has been shown that antibodies can neutralize the virus even after its attachment to the host cell (Vrijsen et al.,

1993; Armstrong et al., 1995). It is hypothesised that neutralization by antibody binding following virus-attachment may be due to the appearance of new epitopes exposed or formed in consequence of the interaction between the virus and the cell receptor (Kjellen 1985). In the case of HIV-1, it was demonstrated that changes in the conformations of the envelope glycoproteins during the fusion process between viral envelope and host cell membrane, lead to the exposure of previously occult epitopes or *de novo* formation of neo-epitopes (Montefiori and Moore 1999). Antisera raised against such neo-epitopes neutralized an array of diverse HIV-1 primary isolates as well (Montefiori and Moore 1999). The ability of some of the antibodies to neutralize both before and after attachment to the host cell may indicate that these antibodies may be more important for protective immunity *in-vivo* compared to antibodies that neutralize only before attachment (Dimmock 1995).

1.2.2.1.3. Interference with uncoating

Inhibition of fusion of enveloped viruses to the plasma membrane can also lead to neutralization. Enveloped viruses belonging to the *Retroviridae, Herpesviridae,* and *Paramyxoviridae* families enter the host cell by fusion of the virion envelope with the plasma membrane (Dimmock 1995). For mAbs specific to gp120, the outer domain of the envelope protein of HIV-1 did not inhibit the virus from binding to its receptor CD4 but neutralized the virus by inhibiting primary fusion and uncoating (McInerney et al., 1997). The number of antibody molecules per virion needed for this type of neutralization is yet to be determined. Some enveloped viruses (e.g., Influenza A virus) are taken up by the cell through adsorptive endocytosis within a vesicle which is subsequently converted into an endosome.

Acidification of the endosome can lead to a series of conformational changes including the activation of fusion peptides. This will result in the fusion of the viral envelope with the endosomal wall and subsequent uncoating of the virus (Bullough et al., 1994). Antibodies can interfere with the requisite fusogenic protein-protein interactions or with conformational changes that are instrumental to the fusion process. Alternatively, antibodies can simply obstruct contact between the two lipid membranes. It has been shown that some of the specific mAbs to Influenza A virus can neutralize the virus by inhibiting the endosomal fusion stage of the infectious pathway (Outlaw et al., 1993). Virus neutralization of poliovirus by various neutralizing mAbs involves the prevention of the cell-mediated conversion of virus to 135S and 80S particles in the acidic endosome leading to the inhibition of uncoating of the virus (Vrijsen et al., 1993).

1.2.2.1.4. Interference with stages of infection subsequent to primary uncoating

Antibodies can also neutralize the virus without having any effect on the attachment, uptake, uncoating of the virion, and internalization of subvirion particles by the cell. This mechanism of virus neutralization has been shown in the case of mAb specific to the V3 loop of gp120 of HIV-1 (Armstrong et al., 1996). This mAb inhibited a stage subsequent to primary uncoating, thus the viral core enters the cytoplasm, leaving behind the viral envelope and the bound mAb as an integral part of the plasma membrane (Armstrong et al., 1996). Particularly intriguing examples of putative post-fusion neutralization are provided by influenza virus and antibodies to its haemagglutinin protein (Armstrong and Dimmock 1992). Antibodies to haemagglutinin protein can neutralize the virus at high concentrations by blocking viral

attachment to the receptor. Lower concentrations of antibodies neutralized the virus by changing the distribution of viral nucleoprotein from its normal concentration in the nucleus to a perinuclear, cytoplasmic localization (Armstrong and Dimmock 1992). In influenza virus infection there is also evidence that secondary uncoating takes place and the virion RNP reaches its proper location at the cell nucleus, but fails to initiates transcription. Detergent uncoating of the neutralized virus showed that the transcriptase had not lost activity and could still recognize and use capped mRNAs as primers, and that the RNA template was intact (Possee et al., 1982; Taylor et al., 1985). How neutralizing antibodies can affect post-internalization events is not known. It was hypothesized that antibodies send a transmembrane signal through the envelope and abrogate the functionality of the viral nucleocapsid (Armstrong et al., 1996).

1.2.2.2. Biological relevance of virus neutralization

In-vitro, virus neutralization assays are conducted by mixing serial dilutions of serum or mAb with virus, incubating, and assaying for residual infectivity with cultured cells, embryonated eggs, or animals. When cell culture is used, the endpoint is defined as the highest dilution of the antiserum that inhibits the development of CPE. In embryonated eggs or animals, end point can be assessed by the development of disease or death of the test unit (Flint et al., 2000). Neutralizing antibodies define type-specific antigens on the virus particle. Therefore, neutralization tests have been invaluable for serotyping the virus. In addition, information on the antigenic composition of a virus can be used to understand the immune response in order to design vaccination strategies. The use of mAbs in neutralization assays

permits mapping of neutralizing epitopes on a virus particle or the amino acid sequence that gives rise to neutralizing antibodies (Flint et al., 2000). The protective activities of antibodies *in-vivo* is determined by the passive transfer of immune sera or mAbs to a naïve animal, which during viral challenge a protective outcome is observed. For many viruses, animal models, and challenge routes, this approach has consistently shown a good correlation between the protection that is achieved *in-vivo* and antibody or serum neutralizing activity measured *in-vitro* (Parren et al., 2001). However, neutralization may not be the only protective mechanism of antibody *in-vivo.* It may include effector functions triggered by the Fc domain of the antibody such as phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) (Kedar et al. 1977; Burton 2002).

Neutralization is not solely the result of virus-antibody interaction, and can under certain circumstances be determined by the host cell as well. That is, some combinations of virus and antibody result in neutralization in one cell type but give no neutralization in another (Dimmock 1995). The practical implications of cell-dependent neutralization are important in view of the necessity to extrapolate from experiments in cultured cells *in-vitro* to the situation *in-vivo*. The antibodies which neutralize *in-vitro* may or may not protect *in-vivo* (Dimmock 1993). The protection may be dependent on the host cell, on complement, or on the appropriate Fc receptors in the membranes of cells which have the ability to destroy the virus. Once the difference between the situations in the laboratory and the whole animal is recognized, it is possible to test for neutralization *in-vitro* using primary cells, providing that the host cell type or tissue is known, available, and can be maintained in the laboratory as a

model for activity *in-vivo* (Dimmock 1995). For example, Influenza A virus proved to be neutralized by the same mechanism in tracheal organ cultures as in BHK cells (Outlaw et al., 1990a and b). In order to properly extrapolate to *in-vitro* situations, cell types used to investigate mechanisms of neutralization must be similar to those considered important *in-vivo* (Klasse and Sattentau 2001).

1.2.3. Antibody dependent enhancement (ADE) of virus infectivity

Despite major vaccine successes of the past century in the prevention of viral diseases, such as those caused by smallpox and poliovirus, many viruses remain refractory to vaccine-induced immune control. There are cases in which vaccination has been reported to predispose individuals to exaggerated or enhanced disease upon exposure to virus. This was first observed in 1960s in epidemiological studies of infections caused by dengue or respiratory syncytial viruses (RSV) and the term "antibody-dependent enhancement" (ADE) of viral infection was formally introduced to describe the phenomenon of immune-enhanced disease (Kapikian et al., 1969; Kim et al., 1969; Halstead et al., 1969, 1970). The paradoxical phenomenon of ADE of virus infectivity was first described in the mid 1960s by investigators performing plaque reduction neutralization tests with several flaviviruses and rabbitpox virus in primary chicken embryo fibroblast cultures (Hawkes 1964; Hawkes and Lafferty 1967). It was shown that the replication of some viruses was enhanced rather than inhibited in the presence of low titer of hyperimmune serum. These studies were confirmed by subsequent studies on West Nile virus (Peiris and Porterfield 1979; Cardosa et al., 1983), yellow fever virus (Schlesinger and Brandriss 1981), Japanese encephalitis virus (Cecilia and

Ghosh 1988), and Murray Valley encephalitis (Kliks and Halstead 1983, Wallace et al., 2003). Since then ADE has been demonstrated for viruses from several other families (Table 1.1).

Some of these viruses represent a significant human health threat, and others are of veterinary importance. Although many of these viruses demonstrating ADE *in-vitro* have also been associated with higher morbidity or mortality in case of prior immunity, the link between *in-vitro* observations of ADE and the *in-vivo* clinical phenomenon of disease enhancement is difficult to establish (Sullivan et al., 2001). Most of these viruses show certain common features including their ability to; (a) replicate in part or exclusively in macrophages, (b) induce the production of large amounts of antibodies that poorly neutralize even homologous virus, and (c) cause persistent infections which are commonly characterized by viremia of long duration (Tirado and Yoon 2003). Antigenic diversity among isolates is also a common feature of these viruses, which renders them partially resistant to neutralization by antibodies raised against heterologous isolates (Tirado and Yoon 2003).

1.2.3.1. Fc receptor-mediated ADE

The precise mechanism of ADE of virus infection is not completely understood. It is generally assumed that increased yields of virus are primarily due to a greater number of susceptible cells being infected (Gimenez et al., 1989; Corapi et al., 1992; Olsen et al., 1992).

Table 1.1. Viruses for which ADE of infectivity has been demonstrated

Family	Virus	Reference(s)
Arenaviridae	Lassa fever virus	Lewis et al., 1988
Bunyaviridae	Rift Valley fever virus	Peiris and Porterfield 1981
Coronaviridae	Feline infectious peritonitis virus	Hohdatsu et al., 1991; Olsen et al., 1992
Filoviridae	Ebola virus	Takada et al., 2003
Flaviviridae	Dengue virus West Nile virus Yellow fever virus Japanes encephalitis virus Murray Valley encephalitis	Halstead et al., 1969,1970 Peiris and Porterfield 1979; Schlesinger and Brandriss 1981 Cecilia and Ghosh 1988 Kliks and Halstead 1983; Wallace et al., 2003
Herpeseviridae	Mouse cytomegalovirus	Inada et al., 1985
Orthomyxoviridae	Influenza A Infectious salmon anaemia virus	Ochiai et al., 1992 This thesis
Paramyxoviridae	Respiratory syncytial virus	Kapikian et al., 1969; Kim et al., 1969; Gimenez 1990
Parvoviridae	Aleutian disease virus	Porter et al., 1980; Kano et al., 1993
Picornaviridae	Coxsackie virus B3	Girn et al., 2002
Reoviridae	Mammalian reovirus, 1 and 3	Burstin et al., 1983
Retroviridae	Human immunodeficiency virus Equine infectious anaemia virus, Caprine arthritis-encephalitis virus Simian immunodeficiency virus	Robinson et al., 1988; Takeda et al., 1988; Montefiori et al., 1989; Jolly et al., 1990; Wang et al., 1994; Fust 1997; Villinger et al., 2003
Rhabdoviridae	Rabies virus	Blancou et al.,1980, Prabhakar and Nathanson 1981
Togaviridae	Ross River virus	Lidbury and Mahalingam 2000

This increase in the infection rate of cells is shown to be mediated mostly by Fc receptors (FcR), that facilitate the uptake of virus-antibody complexes by target cells through FcR-mediated endocytosis. The FcR-mediated mechanism of ADE of virus infection was first suggested by Halstead and O'Rourke (1977) who reported that $F(ab')_2$ fragments prepared from IgG did not enhance infection of dengue virus in human peripheral blood leukocytes.

Generally, interaction between virus-antibody complexes and FcR on monocytes or macrophages induce signal transduction, resulting in phagocytosis, release of cytokines, a superoxide burst, and antibody-dependent cell cytotoxicity (Roite et al., 1985). It is not known how virus evades this antiviral host response and results in enhanced infection. It is possible that these viruses have the ability to modulate antiviral mechanisms of the cells by utilizing their own products or by interfering with the metabolic pathways of the cells (Tirado and Yoon 2003). It is also assumed that infections by virus-antibody complexes are restricted to immunologically immature sub-populations of the cells (Halstead 1982). Halstead and co-workers found that human monocytes became increasingly resistant to infection when cultured more than a day prior to being infected with dengue virus-antibody mixture (Halstead and O'Rourke 1977). Restriction of viral infection to immunologically immature cells was also demonstrated in mice that were persistently infected with lactate dehydrogenase virus (Stuekemann et al., 1982). Study with Ross river virus (RRV), the causative agent for epidemic polyarthritis in human, suggested that the cellular environment may become less antiviral following FcR-mediated entry and replication of the virus (Lidbury and Mahalingam 2000). In this case, production of certain transcriptional factors

for antiviral genes in murine macrophages were inhibited when the cells were infected with the virus through FcR-mediated pathway instead of natural receptors of the virus on the cell. Investigation of Fc- mediated infection pathway found that RRV was able to suppress the transcription and translation of key antiviral genes (tumor necrosis factor and inducible nitric oxide synthase) in LPS-stimulated macrophages by disrupting the transcription into mRNA of the genes coding for the associated transcription factors IRF-1 and NF-kappaB (Lidbury and Mahalingam 2000).

Studies have shown that other mechanisms could also account for ADE. A recent study on ADE of Ebola virus infection showed that antibodies binding to the envelope proteins lead to the binding of complement (C1) with the Fc portion of the antibody. The resultant complex of the virus, antibodies, and complement binds to the complement ligand at the cell surface, promoting either binding of the virus to Ebola virus-specific receptors or endocytosis of the virus by intracellular signalling via complement ligands (Takada et al., 2003). In the case of HIV-1, at least four mechanisms of ADE have been described. The first mechanism is a CD4 dependent, FcR-mediated ADE. In one study, a macrophage-like cell line lacking CD4, a receptor for HIV-1, did not show ADE. This observation implied that attachment of antibody coated virus to Fc receptors increased the interaction of gp120 (receptor binding protein of HIV-1) with its specific receptor (CD4) leading to enhancement of infection (Perno et al., 1990; Jouault et al., 1991). The second mechanism is a CD4-independent FcR-mediated ADE. It has been shown that under some conditions, ADE can circumvent the use of HIV receptors. Studies using cultures of human primary peripheral blood mononuclear

cells showed that blocking of FcR alone eliminated the occurrence of enhanced infection by human or chimpanzee immune sera, confirming the importance of FcR in HIV-1 ADE (Homsy et al., 1989). The third mechanism is a complement-mediated ADE. Robinson et al. (1989) and Prohazka et al. (1997) showed that enhancement of HIV-1 infection can proceed by the alternative pathway of complement, whereby HIV-specific immunoglobulin and complement facilitate virus attachment and uptake by binding to complement receptors. However there is also evidence that complement-enhanced HIV-1 infection can proceed in the absence of HIV-specific antibodies and/or CD4 (Boyer et al., 1991, 1992). The fourth mechanism is an Fc receptor and complement receptor-independent ADE. Studies using purified antibody preparations and permissive cells without FcR showed that ADE can occur in the absence of complement and FcR (Schutten et al., 1995, 1997). In this case antibody binding to gp120 of HIV-1 induces conformational changes in the envelope glycoproteins that are thought to resemble virus receptor activated modifications of gp120 structure that enable association with chemokine co-receptors to promote downstream membrane-fusion events and enhancement of infection (Sullivan 2001).

1.2.3.2. Antibodies and epitopes mediating ADE

Enhancement of virus infection has been demonstrated using antibodies from various sources including polyclonal antisera, mAbs, and antibodies purified from antisera. Experiments using fractionated IgG and IgM antibodies showed that only the IgG fraction caused ADE of dengue virus infection (Halstead and O'Rourke 1977). Similar observations were made in the case of FIP virus where only IgG class mAbs enhanced virus infection (Olsen et al.,

1992). In the case of Ebola virus where complement plays an important role in ADE, it is suggested however, that IgM antibodies can contribute to the ADE due to the fact that complement binds more efficiently to polymeric IgM than to the monomeric IgG antibodies (Borsos 1992; Takada et al., 2003). It is not known whether other isotypes of antibodies (IgA, IgD, and IgE) can mediate ADE. Different subtypes of IgG have also been evaluated for their ability to mediate ADE of virus infection. IgG1, IgG2a, and IgG2b mAbs specific for envelope protein of dengue virus are reported to enhance infection in cells with Fc receptors (Henchal et al., 1985; Morens et al., 1987). No information is available about the role of IgG3 in ADE of dengue virus infection. In the case of FIP virus, most of the enhancing antibodies were of IgG2a subtype (Corapi et al., 1992). The difference in immunoglobulin subclass between neutralizing mAbs that induced ADE and those that did not induce ADE suggests that there may be a restriction in the immunoglobulin subclasses capable of mediating ADE (Corapi et al., 1992). Use of feline macrophages also suggests that the difference in the ability of FIP virus-specific murine IgG isotype to mediate ADE of infection is due to differences in the binding affinity of murine isotypes to FcR on feline macrophages (Hohdatsu et al., 1994).

Ochiai et al. (1988) demonstrated the antibody-enhanced internalization and growth of Influenza A virus NWS (H1N1) in the cultured murine macrophage-like cell line in the presence of sub-neutralizing antiviral IgG. Their experiments revealed that the intracellular pathways of the virus after internalization via Fc receptors or viral receptors are similar. HA and NA of Influenza A virus induce antibodies that enhance uptake of viruses via Fc

receptors in mammalian macrophage-like cell lines (Tamura et al., 1991). Despite the drift of neutralizing antigen of Influenza A virus strains within a subtype, cross-reactive, non-neutralizing antibodies persisted and they enhanced virus uptake by FcR bearing cells. Therefore, it is suggested that the drift of neutralizing antigens occurred more quickly than the drift of infection-enhancing antigens during the evolution of virus strains of influenza H3 subtype (Tamura et al., 1991). Distinct epitopes of Influenza A HA that induce neutralizing and/or enhancing antibodies have also been identified using monoclonal antibodies (Tamura et al., 1993).

Studies using mAbs showed that ADE is dependent on the epitopes and is not solely the result of the concentration of the virus specific antibodies (Halstead et al., 1984). Since viruses in the same genus or family share common antigenic determinants, ADE of virus infection can be mediated by antibodies raised not only against heterologous strain but also against different serotypes of the viruses or even against closely related viruses in the same genus or family. Dengue virus infection can be enhanced by antisera raised against heterologous serotypes of dengue virus and also by antisera specific for other flaviviruses suggesting that serotype specific as well as flavivirus-cross reactive epitopes are associated with ADE of dengue virus infection (Halstead and O'Rourke 1977; Halstead et al., 1984). Tamura et al. (1994) showed that infection by Influenza A virus can be augmented by pretreating the virus with antisera raised against different subtypes of the virus. Similarly, infection of feline peritoneal macrophages by FIP virus was enhanced by mAbs generated against transmissible gastroenteritis virus of swine, which also belongs to the *Coronaviridae*

family (Olsen and Scott 1993). In their studies on ADE of FIP virus, Olsen and co-workers (1992) were able to categorize the mAbs that represented the epitopes of the envelope S protein into three groups according to their ability to; (a) only neutralize, (b) both neutralize and enhance, and (c) only enhance FIP virus infection. Furthermore, mAbs with specificity for different FIP virus antigenic determinants varied in their ability to enhance virus infection, suggesting that epitopes are strongly or weakly associated with ADE of infection (Olsen et al., 1992; Corapi et al., 1992). These findings also indicate that it may be possible to develop vaccines with strong neutralizing and weak ADE inducing characteristics.

1.2.3.3. ADE determination assays

Although *in-vivo* assays may have more clinical relevance, *in-vitro* assays utilizing various sources of virus specific antibody, such as antisera, mAbs, or fractionated immunoglobulins, are generally performed to determine the ability of antibody to enhance specific virus infections (Halstead et al., 1982, Tirado and Yoon 2003). Either increased production of progeny virus or the proportion of cells being infected after exposure to virus-antibody mixtures is assessed in *in-vitro* studies of ADE. The virus yield is usually determined by microtiter infectivity assay or plaque assay using permissive cell lines (Halstead et al., 1982). Various assays including fluorescent microscopy (Olsen et al., 1992) and *in-situ* hybridization (Olsen et al., 1993) are used to measure the proportion of cells infected with the virus. Morens and Halstead (1990) proposed the following criteria in order to verify true ADE: (i) a statistically significant increase in the virus production as measured by quantitative assays at selected time points on the one step growth curve, (ii) serial dilution

of the pre-incubated antibody source must produce an 'enhancement profile' of rising, peaking, and declining production of infectious virions over at least a 10^3-fold dilution range, (iii) the dilution of the antibody source at which ADE is maximal must be related to its reactivity in other serological tests that measure envelope binding (iv) ADE should be detected with different antibody sources and virus strains tested over a range of multiplicity of infection (m.o.i.), and lastly (v) other causes of increased virus production must be ruled out, including a stabilizing or stimulating effect of the antibody source on the infected cell, prevention of thermal decay of virus by antibody, and competition for cell receptors and antibodies by either non-infectious or soluble or membrane antigens in the virus stock.

The general approach for the demonstration of ADE *in-vivo* is to challenge susceptible host animals with the target virus after injecting them with antibodies (purified antibody or antiserum). The level of virus replication is then monitored and compared to animals that did not receive antibody. Halstead (1979) injected rhesus monkeys intravenously with human cord-blood containing anti-dengue virus antibody and immediately challenged them with dengue virus and demonstrated that the monkeys that were injected with dengue virus antibody developed higher levels of viremia for a longer period than control monkeys. Most recently, *in-vivo* ADE has been demonstrated in the case of Murray Valley encephalitis virus where passively immunized mice showed increased viremia and mortality following viral challenge (Wallace et al., 2003).

1.2.3.4. Biological significance of ADE

Many of the viruses demonstrating ADE *in-vitro* have also been associated with higher morbidity or mortality in case of prior immunity. In these cases, presence of antibodies increased the susceptibility to subsequent viral infections and/or exacerbated the severity of clinical disease by virus challenge in vaccinated individuals (Tirado and Yoon 2003). The association between ADE of infection and the severity of dengue haemorrhagic fever has been extensively studied. This association was first observed by Halstead and co-workers who observed that the severity of dengue fever was significantly greater in children with maternal antibody specific for dengue virus than in children with no dengue virus-specific maternal antibody (Halstead 1970; Halstead et al., 1970). In another study, infants with maternally acquired respiratory syncytial virus antibody were not only susceptible to virus infection, but the rate of severe infection was higher in these infants when compared to infants without maternal antibodies (Chanock et al., 1970).

Antibody-dependent enhancement has been incriminated as a disease-enhancing factor of FIP. Cats with active or maternal immunity to FIP virus often develop an accelerated and more fulminant disease following challenge with FIP virus compared to seronegative cats (Weiss and Scott 1981a, b, c). Immune-mediated disease enhancement has been demonstrated in kittens that had vaccine-derived humoral immunity directed against the spike protein of FIP virus which resulted in earlier death than control animals (Vennema et al., 1990).

Aleutian disease virus (ADV) causes a fatal glomerulonephritis in mink due to deposition of soluble immune complexes on renal glomerular membrane or wall of capillary blood vessel. This parvovirus circulates in the blood mainly as immune complexes, which are fully infectious, both *in-vivo* and *in-vitro* (Porter et al., 1980). There is also strong evidence suggesting that ADE plays an important role in the pathogenesis of ADV. It has been demonstrated that infection of mink peritoneal macrophages by ADV can be enhanced by anti-ADV antibodies (Kano et al., 1993). Moreover, in a vaccination trial using inactivated ADV, eight out of ten vaccinated animals developed severe Aleutian disease as compared to the control animals (Porter et al., 1972). Disease enhancement due to vaccine-induced antibodies has also been demonstrated in the case of equine infectious anaemia virus (Issel et al., 1992), and exacerbation of disease severity in vaccinated animals has also been observed for other lentiviruses (McGuire et al., 1986).

The presence of non-neutralizing antibodies capable of facilitating virus entry into macrophages, cells expressing Fc, or complement receptors may contribute to the persistence by creating a virus reservoir in macrophages. ADE of virus infection can be a major obstacle to the development of effective vaccines for disease control. In those cases, identification of viral epitopes associated with ADE and neutralization is important. Potential risk may be minimized by avoiding enhancing epitopes in recombinant subunit or DNA vaccine preparations. Clear understanding of the cellular events during ADE of virus infection is also essential for effective intervention.

1.2.4. Infectious salmon anaemia virus (ISAV)

1.2.4.1. Incidence and geographic distribution of ISA

Infectious salmon anaemia (ISA) is a fatal disease of farmed Atlantic salmon caused by a new orthomyxovirus, namely infectious salmon anaemia virus (ISAV). ISA is characterized by severe anaemia and high mortality in farmed Atlantic Salmon, *Salmo salar* (Nylund et al., 1995). The disease has occurred in Norway since 1984 (Jarp and Karlsen, 1997), but the virus may have been present in Norwegian salmon farms as early as 1977 or 1978 (Devold et al., 2001; Nylund et al., 1995a). The Office International des Epizooties (OIE, World Animal Health Organization) officially recognized the disease in 1990 and named it ISA (Håstein 2003). The first ISA outbreak to occur outside of Norway was in New Brunswick, Canada, in 1996 (Byrne et al., 1998; Mullins et al., 1998), although there is anecdotal evidence indicating that the virus may have been present in the Bay of Fundy in 1995. ISA outbreaks have also occurred in Scotland, U.K., in 1998 and 1999 (Bricknell et al., 1998; Rodger et al., 1999; Murray 2003), in the Faroe Islands, Denmark, since 2000 (Anon. 2000; Lyngøy 2003), in Maine, USA, since 2001 (Bouchard et al., 2001), and in Nova Scotia, Canada, in 2000 (Kibenge et al., 2001b). Laboratory data on tissues and sera from farmed coho salmon (*Oncorhynchus kisutch*) support the existence of ISAV in Chile as well (Kibenge et al., 2001a, 2002). The virus has also been detected in apparently healthy farmed rainbow trout (*Oncorhynchus mykiss*) in Ireland in 2002 (Anon. 2002), and in asymptomatic wild fish [sea trout (*Salmo trutta trutta*), Atlantic salmon, and brown trout (*Salmo trutta* L)] from the same geographical locations with Atlantic salmon farms experiencing clinical ISA in Scotland (Raynard et al., 2001a; Cunningham et al., 2003).

1.2.4.2. Properties and structure of ISAV

Infectious salmon anaemia virus is now classified in the family *Orthomyxoviridae* (Krossøy et al., 1999), genus *Isavirus* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). ISAV shares several morphological, biochemical, and physiochemical features with those of influenza viruses. It is the only species of the genus *Isavirus*, one of the five genera of the *Orthomyxoviridae* family. Other genera include *Influenzavirus A, Influenzavirus B, Influenzavirus C, and Thogotovirus.* The structural properties of the genus *Isavirus* include enveloped virions of 90-140 nm diameter (Dannevig et al., 1995; Nylund et al., 1995b) with a genome composed of eight segments of linear, single-stranded negative sense RNA ranging in length from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb (Clouthier et al., 2002). All of the eight RNA genomic segments of ISAV have been sequenced (Mjaaland et al., 1997; Krossøy et al., 1999, 2001a; Snow et al., 2001a, 2003; Rimstad et al., 2001; Ritchie et al., 2001a, 2002; Clouthier et al., 2002). Comparison of the ISAV proteins with those of other orthomyxoviruses revealed low amino acid identity values, between <13% and <25% (Krossøy et al., 1999; Kibenge et al., 2001b; Snow and Cunningham, 2001; Ritchie et al., 2002), and the order of the genome segments encoding the proteins in ISAV appears to differ from those of influenza viruses (Table 1.2).

The buoyant density of the complete virus particles in sucrose and cesium chloride gradients is 1.18 g/mL (Falk et al., 1997). The virus is sensitive to chloroform, ether, heat, UV irradiation, and extremes of pH (Kristin and Rimstad 2001; Falk et al., 1997; Mjlaand et al., 1997; Thorud 1991).

Table 1.2. Comparison of genome coding assignments for ISAV and Influenzavirus A

	Gene products[1]	
Genome segment	Isavirus	Influenzavirus A
1	PB2 (84K)[2]	PB2 (96K)
2	PB1 (84K)	PB1 (87K)
3	NP (77K)	PA (85K)
4	PA (71K)	HA1(48K), HA2(29K)
5	Unknown (47K)	NP (50-60K)
6	HE (42K)	NA (48-63K)
7	Unknown (31K), NEP (17K)	M1(25K), M2(15K)
8	M1 (24K), NS1 (16K)	NS1(25K), NS2 (12K)

[1] Gene products: PB2, PB1, and PA: Polymerase; NP: Nucleoprotein; HE: (haemaglutinin esterase in ISAV; Falk et al., 2004); HA: Haemagglutinin (two subunits in Influenzavirus A); NA: Neuraminidase; M1: Matrix protein; M2: Ion channel protein; NS1: Nonstructural protein 1; NS2: Nonstructural protein 2 (also known as Nuclear export protein, NEP).

[2] Numbers in parenthesis are the observed molecular weights (in kilodaltons). *Isavirus* data are from Kibenge et al. (2004). *Influenzavirus A* data are from Cox et al. (2000).

Exposure to 56°C for five minutes or pH 4.0 for 30 minutes resulted in complete loss of virus infectivity. The virus has been shown to survive in seawater for 20 hours at 6°C and in fish tissues (blood and kidney) for 4 days at 6° C (Nylund et al., 1995b). This difference is not unexpected considering the cell-associated nature of the virus (Kibenge et al., 2000). A 1000-fold reduction in the infectivity titer in freshwater was observed upon exposure to ultraviolet irradiation at a dose of 6.4-9.4 J/m^2, while in wastewater the equivalent inactivation of the virus required a dose of 56-88 J/m^2 (Kristin and Rimstad 2001). Chemicals such as chloroquine, ammonium chloride, and bafilomycin A1 inhibit ISAV replication in cell culture (Eliassen et al., 2000). These agents elevate the intraluminal pH in the endosomes, thereby inhibiting the activation of the membrane fusion protein required for the release of viral ribonucleoproteins (RNPs) into the cytoplasm.

1.2.4.3. ISAV genome coding assignments

Purified ISAV was shown to contain four major polypeptides with estimated molecular size of 71, 53, 43/46, and 24/26.5 kDa (Falk et al., 1997; Kibenge et al., 2000). Most recent studies analyzed four major structural proteins of ISAV and assigned molecular weights of 66, 50, 42, and 22 kDa, respectively, to the four major structural proteins of ISAV. This included two glyosylated proteins with estimated molecular masses of 42 and 50 kDa, one 66 kDa phosphoprotein, and one 22 kDa protein (Falk et al., 2004). This study showed that 42 and 50 kDa proteins were glycosylated using metabolic labeling with tritiated mannose, lectin binding, and deglycosylation experiments. Metabolic labeling with ^{32}P demonstrated that 66 kDa protein was a phosphoprylated structural protein (Falk et al., 2004). When

purified virus was solubilized by nonionic detergent NP-40 it was observed that 50, 42, and 22 kDa proteins were soluble and 66 kDa protein was insoluble. It was also shown that 22 kDa protein is a late protein accumulating in the nucleus. Based on these observations, Falk et al. (2004) concluded that 42 and 50 kDa proteins are surface proteins, 66 kDa phosphoprotein is the NP protein, and 22 kD protein is the matrix protein of ISAV. This study also proposed to designate the 42 kDa as haemagglutinin esterase (HE) protein (Falk et al., 2004).

Although nucleotide and amino acid sequence information is publicly available for all eight ISAV genome segments, the identities of the proteins encoded by most of the segments have not been determined. Protein expression has only been described for ISAV RNA segments 1 and 6 which encode the putative PB2 (Snow et al., 2003) and haemagglutinin (Rimstad et al., 2001; Krossøy et al., 2001a) proteins, respectively. The genomic segment 1 of ISAV encodes the putative polymerase (PB2) protein. The putative PB2 protein was shown to contain a bipartite nuclear localization signal in the C-terminus of the protein and when expressed in fusion with a green fluorescent protein (GFP) was shown to exhibit nuclear localization in fish cell line CHSE-214 (Snow et al., 2003). Krossoy et al. (1999) described the sequence from segment 2 and suggested that this segment encodes one protein with motifs that are conserved across the *Orthomyxoviridae,* and in RNA-dependent RNA polymerase genes in general. Snow and Cunningham (2001) described segment 3 and analysis of the nucleotide, and predicted amino acid sequences suggested that this segment encoded the NP. The predicted molecular weight of the gene product, 71 kDa, correlated

with the previous estimates of structural proteins (Falk et al., 1997; Kibenge et al., 2000). Ritchie et al. (2001) also described the sequence of segment 3 and included segment 4 as a possible polymerase-encoding gene. Genomic RNA segment 5 is assumed to encode a surface glycoprotein as it carries a hydrophobic signal sequence of 17 amino acids and potential glycosylation sites (Clouthier et al., 2002). RNA segment 6 of ISAV encode the putative haemagglutinin protein (Rimstad et al., 2001; Krossøy et al., 2001a, Falk et al., 2003) which was recently renamed as HE since viral esterase is located with the haemagglutinin on the same protein (Falk et al., 2004). This finding was confirmed by the demonstration of acetylesterase activity in affinity-purified haemagglutinin preparations (Falk et al., 2004). The haemagglutinin protein was recognized by monoclonal antibody 3H6F8 (Falk et al., 1998) directed against ISAV haemagglutinin, and expression of the protein in either insect cells or SHK-1 cell line demonstrated its hemadsorptive properties for salmon erythrocytes (Rimstad et al., 2001; Krossoy et al., 2001). The reported estimated molecular mass of the haemagglutinin protein varies from 46 kDa (Kibenge et al., 2000) to 43 kDa (Falk et al., 1997) to 42 kDa (Falk et al., 2004) to 38 kDa (Griffiths et al., 2001). The molecular mass of haemagglutinin protein predicted from the gene sequence is similar to that observed by SDS-PAGE and Western blotting using purified virus preparation as antigen. Therefore, it was suggested that, unlike in influenza viruses HA, the ISAV haemagglutinin protein did not appear to be cleaved post-translationally (Krossoy et al., 2001).

The gene-protein assignment for viral RNA segment 7 and 8 is controversial as each has two open reading frames (ORFs) capable of encoding proteins of sizes corresponding to M1 and

or NS1 and NEP proteins. The segment 7 encoded proteins were considered non-structural proteins since they failed to react by Western blotting with a polyclonal rabbit antiserum raised against purified ISAV (Biering et al., 2002). The segment 8 ORF of ISAV was reported to encode the 24 kDa protein, one of the major structural proteins of ISAV, although the identity of this protein was not determined (Biering et al., 2002). Our laboratory has studied the gene-coding assignments of ISAV by individually expressing the open reading frames (ORFs) in the eight RNA segments *in-vitro* with rabbit reticulocyte lysates and analyzing their translation products by immunoprecipitation with rabbit antiserum to purified whole virus. The products of segments 1-7 and of ORF1 in segment 8 were immunoprecipitated suggesting that they are structural viral proteins. The segment 8 ORF2 product was not immunoprecipitated and is probably nonstructural (Kibenge et al., 2004). These findings contradict some of the presentations by Cunningham and Snow (2003) about the genetics of ISAV. Thus the ISAV genome appears to encode at least 10 proteins, of which nine are structural and one is nonstructural (Table 1.2).

1.2.4.4. ISAV strain variation

The concept of ISAV strain variation has gained wide acceptance since Blake et al. (1999) first reported partial genomic sequence data of segments 2 and 8 and showed significant differences between Canadian and Norwegian isolates (Cunningham and Snow, 2000; Kibenge et al., 2000a; Inglis et al., 2000; Krossøy et al., 2001b; Ritchie et al., 2001b). ISAV isolates could be grouped into two phenotypes based on their ability to grow and cause CPE in CHSE-214 cells (Kibenge et al., 2000a; 2001b), but the molecular basis for this

phenotypic variation has not yet been determined. Different ISAV isolates also vary in the molecular sizes of their polypeptides (Kibenge et al., 2000a) as do influenza viruses (Kendal, 1975). Most recently, Cook et al. (2003) have reported an assay utilizing RT-PCR and denaturing gradient gel electrophoresis (DGGE) (Myers et al., 1987) that is capable of providing quick and reliable typing of ISAV isolates. However, to date there has been little success to show whether the genetic variations of ISAV are associated with any phenotype of the virus such as antigenic type, serotype, pathotype or virulence.

Alignment of amino acid sequences of the haemagglutinin protein of different ISAV isolates revealed a highly polymorphic region (HPR) in the C-terminal part of the protein (Rimstad et al., 2001) that was suggested to be a useful epidemiological marker of the virus (Krossøy et al., 2001a). In a more extensive analysis using 32 different ISAV isolates, this HPR was found to consist of a 4-16 amino acid deletion in all isolates relative to Canadian ISAV isolate RPC/NB-980 280-2, leading Kibenge et al. (2001b) to suggest that the archetypal ISAV was probably of Canadian origin. A recent analysis of 62 ISAV haemagglutinin sequences in the GenBank database confirmed that there are two haemagglutinin genotypes of ISAV, with the HPR spanning haemagglutinin residues 338-367 (Marshall 2003). Because these genotypes correlated to antigenic groupings, they were designated haemagglutinin subtypes (Kibenge et al., 2001b). The two haemagglutinin subtypes of ISAV have nucleotide sequence identities of ≤80.4% and amino acid sequence identities of ≤88.2% whereas within each subtype, both sequence identities are ≤ 90.7% (Marshall 2003). For Influenza A virus which has up to 15 different HA subtypes, amino acid sequence identities for isolates

belonging to different subtypes can range from 25-80% (Nobusawa et al., 1991; Kawaoka et al., 1991; Wright et al., 1995; Skehel and Wiley, 2000). Thus the haemagglutinin protein of ISAV shows less variation than that of influenza A virus HA protein.

Because many more ISAV isolates of the European haemagglutinin genotype have been sequenced compared to isolates of the North American genotype, more variation is evident in the entire haemagglutinin sequence as well as in the HPR of European isolates (Marshall 2003). Devold et al. (2001) recognized 11 HPR groups among 37 European ISAV isolates and suggested that they arose by homologous recombination within the HPRs. However, Mjaaland et al. (2002) who examined twice as many European ISAV isolates disagreed with the recombination theory. These authors proposed that the patterns of variability in the HPR were due to deletions from a longer donor haemagglutinin gene designated HPR0 that they amplified from a normal wild Atlantic salmon (Cunningham et al., 2003). This HPR0 sequence contained 11 more amino acids than found in ISAV isolate RPC/NB-980 280-2 (Kibenge et al., 2001b), and was suggested to be the archetypal ISAV (Cunningham et al., 2003), although the virus could not be isolated in cell culture. Most recently, an HPR0 sequence has also been detected in Atlantic salmon samples in the Bay of Fundy, NB, Canada (Cook et al., 2004).

The antigenic variation of ISAV isolates is correlated with the genetic variation of the haemagglutinin gene, allowing the grouping of ISAV into North American and European haemagglutinin subtypes (Kibenge et al., 2001b). Since the virus neutralization tests used

only antisera to ISAV isolates of the North American haemagglutinin subtype (Kibenge et al., 2001b), antigenic relationships among the European isolates were not however clearly determined. In recent virus neutralization tests in our laboratory, rabbit antiserum to the Scottish ISAV isolate 390/98 of the European haemagglutinin subtype did not neutralize either the Nova Scotian ISAV isolate U5575-1 (Kibenge et al., 2001b) or Norwegian isolate 485/9/97 which are also of European haemagglutinin subtype. Thus presently there are at least three haemagglutinin subtypes of ISAV: (1) North American, (2) European, and (3) isolate U5575-1 (Kibenge et al., 2004). Alignment of deduced amino acid sequences in the HPR of the haemagglutinin protein of selected ISAV isolates identified the putative antigenic motif around the common potential N-glycosylation site at amino acid positions ^{333}NIT^{335} with one mutation site at positions 320 to 323 on one side and a second mutation site at positions 339 to 367, between European and North American subtypes (Marshall 2003). The Nova Scotian ISAV isolate U5575-1 HA protein has an additional unique potential N-glycosylation site at amino acid positions ^{362}NQT^{364} very close to the predicted transmembrane region (amino acid positions 368 to 409) (Marshall 2003). The presence of the additional carbohydrate chain in this region may result in a new epitope accounting for the isolate U5575-1 (and possibly Norwegian isolate 485/9/97) being antigenically distinct from the European (Marshall 2003) subtype. Studies on RNA segment 5 revealed up to four distinct genotypes among 30 ISAV isolates studied (Kibenge et al., 2004). Thus there is significant antigenic variation among ISAV isolates.

1.2.4.5. ISAV replication *in-vitro*

The first indication that ISAV can be grown in cell culture was when Dannevig and Falk (1994) showed that it replicated in primary cultures of leukocytes from Atlantic salmon. The virus yield was however, low, and cell susceptibility was variable. Subsequently, a continuous cell line, SHK-1, was established from a culture of Atlantic salmon head kidney leukocytes (Dannevig et al., 1995a,b). The SHK-1 cell line allowed replication of ISAV with development of CPE (Dannevig et al., 1995a,b, 1997), and moderate infectivity titers of 10^6 to 10^7 $TCID_{50}$/ml (Falk et al., 1997; Kibenge et al., 2000a). This cell line is widely used for primary isolation of ISAV, and is currently the best fish cell line for this purpose. However, it is a very delicate cell line requiring very low split ratios, a complex growth medium, and at higher passages it seems to lose sensitivity for ISAV resulting in poorly defined and slowly developing CPE by some ISAV strains (Falk et al., 1998; Rolland et al., 2003). Furthermore, it has not been possible to isolate virus from some ISA outbreaks using this cell line (Rimstad and Mjaaland, 2002). Bouchard et al. (1999) successfully used the CHSE-214 cell line to directly recover ISAV from clinical specimens. Because this cell line has a split ratio of 1:4, it is useful for large scale preparations of the virus, as in antigen production for use in vaccines or as a diagnostic reagent (Kibenge et al., 2000). Some ISAV isolates are however noncytopathic (Kibenge et al., 2000b) while others do not replicate in this cell line at all (Munir and Kibenge, 2004), thereby limiting its utility in virus isolation (Kibenge et al., 2000a). Moreover, for cytopathic strains, the CPE develops slowly, taking up to 17 days p.i. compared to 12 days p.i. in SHK-1 cells, and the virus yields are also lower ($10^{4.5}$ to $10^{6.5}$ $TCID_{50}$/ml) than on the SHK-1 cell line (Kibenge et al., 2000a). Newer cell lines derived

from Atlantic salmon head kidney leukocytes such as TO (Wergerland & Jakobsen, 2001) and ASK-2 (Devold et al., 2000; Rolland et al., 2003) cell lines develop more easily discernible CPE sooner (by 2-4 days p.i.) than SHK-1 and CHSE-214 cells, give higher virus yields (as high as $10^{9.1}$ $TCID_{50}$/ml, Wergerland and Jakobsen 2001), and require simpler growth media. They still have low split ratios however, and do not recover virus from some ISAV RT-PCR positive samples (Kibenge et al., 2001a), indicating that the currently available fish cell lines are either not sensitive enough or not permissive for all ISAV strains (Kibenge et al., 2004). ISAV also replicates but is noncytopathic in Atlantic salmon (AS) (Sanchez et al., 1993; Sommer and Mennen, 1997) and rainbow trout gill (RTgill-W1) cell lines (Bols et al., 1994; Falk et al., 1997). Virus replication can be detected by indirect fluorescent antibody test (IFAT), but the virus yield is very low; the maximum viral titer reported on these cells was 10^3 $TCID_{50}$/ml (Falk et al., 1997). Virus replication on FH-10, BB, EPC, and BF-2 cell lines has also been attempted, but these cell lines appear to be refractory to ISAV (Byrne et al., 1998; Falk et al., 1997).

The events involved in the replication of ISAV have not been fully explored, but the replication strategy of the virus seems to resemble that of influenza viruses (Hovland et al., 1994; Falk et al., 1997; Mjaaland et al., 1997; Sandvik et al., 2000; Eliassen et al., 2000; Snow et al., 2001a; Rimstad and Mjaaland 2002). The influenza viruses attach via their HA to host-cell receptors terminating in sialic acids (Zambon 2001). Haemagglutination assays with erythrocytes from several fish species and horse have indicated that ISAV uses its haemagglutinin to bind sialic acid receptors on the cell surface (Eliassen et al., 2000; Falk

et al., 1997; Kristiansen et al., 2002). Structurally diverse as well as certain unique sialoglycoproteins have been reported to occur in salmonid species (Iwasaki et al., 1990). Most recent studies showed that ISAV specifically binds to glycoproteins containing 4-O-acetylated sialic acids and it hydrolyzed ISAV receptors on horse and rabbit erythrocytes, indicating that 4-O-acetylated sialic acids represents a receptor determinant for ISAV (Hellebø et al., 2004).

Eliassen et al. (2000) have reported that the initial events in ISAV infection are similar to those of influenza virus infection (Flint et al., 2000; Portela and Digard 2002); they demonstrated the internalization of ISAV into the endosomes and low-pH-dependent fusion of the viral envelope with the endosomal membrane. In the case of influenza virus the acidification of endosome causes conformational change in HA, resulting in the fusion of endosomal and viral membrane. Two critical events occur on uncoating; firstly, acidification of the interior of the virion by M2-mediated channeling of protons causes M1 protein to dissociate from RNP. Secondly, the viral membrane fuses with the endosomal membrane via a low pH-triggered conformational change of the HA to release the RNP into the cytoplasm (Portela and Digard 2002).

Increase in the virus infectivity titer by the treatment of ISAV with trypsin suggested that ISAV replication, similar to that of influenza viruses, may be dependent on the post-translational proteolytic activation of haemagglutinin protein (Falk et al., 1997; Kibenge et al., 2000; Rimstad and Mjaaland 2002). This is not absolutely essential as ISAV replicates

efficiently without trypsin treatment in cell cultures. In Influenza A viruses, amantadine specifically inhibits the M2 ion channel activity and therefore, vRNPs are not released into the cytoplasm. In the case of ISAV, amantadine did not have any effect on viral replication (Falk et al., 1997). Once in the cytoplasm, RNPs migrate to the nucleus and enter through nuclear pores (Portela and Digard 2002). Transcription is activated in the nucleus and requires 'cap-snatching,' that is, excision of host caps to prime viral mRNA. Thus viral mRNAs possess a cap structure of host origin at their 5' termini and are polyadenylated at their 3' ends. The viral genomic segments are replicated and transcribed by the viral RNA dependent RNA polymerase associated with the RNPs. The viral RNA is directly transcribed to mRNA and, in addition, serves as a template for a complimentary copy (cRNA) which itself is the template for new viral RNA (Ludwig et al., 1999; Portela and Digard 2002).

In the late phase, the newly synthesized viral genome is packaged by the NP protein and the resulting RNPs are exported to the cytoplasm. NEP and M1 proteins play important roles in the export of the RNPs into the cytoplasm and then to the plasma membrane. NS1 protein functions as a regulatory factor in the virus infected cell. The NA, M2 and the precursor HA (HA0) proteins follow the exocytotic transport pathway from the endoplasmic reticulum via the Golgi complex and the trans Golgi net work. The mature HA and NA glycoproteins and the non glycosylated M2 are finally inserted into the plasma membrane as trimers (HA) or tetramers (NA, M2), respectively. M1 assembles in patches at the cell membrane. It is thought to associate with the glycoproteins (HA and NA) and recruit the RNPs to the plasma membranes in the late phase of the replication cycle. Finally the RNPs become enveloped

by a cellular bilipid layer carrying the HA, NA and M2 proteins resulting in budding of new virus particles from the apical cell surface (Ludwig et al., 1999; Brown 2000). Although ISAV has been reported to be released by the budding process (Hovland et al., 1994) and HA and RDE activities have been demonstrated on ISAV particles, the exact mechanism involved in the trafficking of vRNPs and other proteins, assembly of progeny virions at the plasma membrane, maturation and exit of the progeny virions, and role of viral proteins in each step of the viral replication have yet to be investigated.

1.2.4.6. Host range of ISAV

ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon (Nylund et al., 1994, 1996; Dannevig et al., 1995a,b; Byrne et al., 1998; Rodger et al., 1998) and asymptomatic infection in feral fish (Raynard et al., 2001a), a situation analogous to avian influenza viruses in domestic poultry and feral birds (Kibenge et al., 2004). The clinical ISA disease can also occur in wild free-ranging Atlantic salmon, but these fish are less susceptible than the farmed Atlantic salmon either due to genetic variation in the two fishes or due to stress caused by management practices on the salmon farms (Nylund et al., 1995b). ISAV may infect and replicate in sea trout, brown trout, rainbow trout, eels (*Anguilla anguilla*), herring (*Clupea harengus*), and Arctic char (*Salvelinus alpinus*) resulting in asymptomatic, probably life-long, carriers of the virus (Nylund et al., 1994; 1995b; 1997; 2002; Nylund and Jakobsen, 1995; Rolland and Nylund, 1999; Snow et al., 2001b; Devold et al., 2001). The Pacific salmonid species, chum (*Oncorhynchus keta*), steelhead (*O. mykiss*), Chinook (*O. tsawytscha*), and coho (*O. kisutch*) were also found to be resistant to

experimental infection with ISAV even with doses as high as 10^8 TCD_{50}/ml that induced 98% mortality in Atlantic salmon (Rolland and Winton 2003). Although attempts to isolate the virus from some of these fishes have not been successful, the viral RNA can be detected by RT-PCR (Devold et al., 2001; Raynard et al., 2001a; Snow et al., 2001b; Nylund et al., 2002). Such subclinical infections in the wild fishery are considered to be the source of virulent strains that cause clinical disease in marine-farmed Atlantic salmon (Murray et al., 2002).

MacLean et al. (2003) surveyed nearly 3,000 non-salmonid marine fishes including alewife (*Alosa pseudoharengus*), American eel (*Anguilla rostrata*), Atlantic herring, Atlantic mackerel (Scomber scombrus), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Pollock (*Pollachius virens*), American shad (*Alosa sapidissima*), and winter flounder (*Pseudopleuronectes americanus*). ISAV was detected only in pollock and cod collected from cages with ISA-diseased salmon. Pollock have been shown to be able to eliminate the virus within a week of experimental infection (Snow et al., 2002). It can be argued that fish species, other than Atlantic salmon, are naturally resistant to the clinical disease and/or that ISAV strains vary in virulence. However, it is more likely that the determinants for the host range of ISAV are multifactorial since Atlantic salmon that recover from clinical ISA as well as subclinically infected trout and herring which are negative on virus isolation attempts (or RT-PCR, McAllister et al., 2003) can transmit ISAV to healthy Atlantic salmon through cohabitation (Nylund and Jakobsen, 1995; Nylund et al., 1997, 2002; McAllister et al., 2003). ISAV does not infect

humans or other mammals, since the virus is inactivated at pH below 4.5 (and human gastric secretions are at pH 2.0), and does not replicate *in-vitro* at temperatures of 25° C or above (Falk et al., 1997), and it does not appear able to replicate in human cells (Anon. 2000b).

1.2.4.7. Pathogenesis of ISAV

Infectious salmon anemia is transmitted from fish to fish by contact with infected fish, parts of infected fish (mucus, blood, viscera, trimmings, muscle, feces), contact with equipment contaminated by infected fish, people who handled infected fish, fish products, contaminated equipment or have been on ISA-infected sites without adequate disinfection (Rolland and Nylund 1998). Sea lice (*Lepeophtheirus salmonis*) can transmit ISAV from infected to susceptible fish (Nylund and Jacobsen 1995). The most probable portal of entry for the virus into the fish is through the gills (Totland et al., 1996). Pillar cells of the gills were the first place where the virus was found to appear after an i.p challenge (Totland et al., 1996). Considerable viral replication occurs within infected fish and the virus is widely disseminated throughout most tissues including mid-kidney, head kidney, liver, spleen, intestine, gills, muscle, and heart (Jones et al., 1999; Rimstad et al., 1999). By electron microscopy, budding of ISAV has been localized to endothelial cells of blood vessels in several organs (Hovland et al., 1994; Nylund 1995). Therefore, endothelial cells may be the target cells for ISAV (Rimstad et al., 1999).

Infectious salmon anemia causes a severe anemia and haemorrhagic lesions in Atlantic salmon. Heamatocrit values of less than 10 have been observed in moribund fish and a

leucopenia seems to develop concomitantly with anemia (Thorud 1991). Infectivity of kidney and liver was present seven days post-infection while a decrease in haematocrit values was observed 14-18 days post-infection (Dannevig et al., 1994). Pathological changes of perisinusoidal interhepatocytic macrophages have been demonstrated by electron microscopical studies 4 days post-infection (Speilberg et al., 1995). Thus, cells and tissues of Atlantic salmon are affected before clinical signs of ISA can be detected. Leucocytes isolated from the head kidney of ISA-infected salmon have been demonstrated to contain ISAV (Dannevig et al., 1994). Studies have shown that changes occur in the leucocyte populations of the spleen and head kidney of Atlantic salmon early in the course of disease. The early and marked infectivity of head kidney leucocytes, their ability to support *in-vitro* propagation of ISAV and their abnormal distribution *in-situ* suggest that head kidney leucocytes are one of the target cells of ISAV (Falk et al., 1995). *In-situ* hybridization studies also showed that ISAV targets endothelial cells, macrophage-like cells or mononuclear leucocytes *in-vivo* (Moneke et al., 2003).

Microscopic pathological changes are characterized by renal interstitial hemorrhage and tubular nephrosis, branchial lamellar and filamental congestion, congestion of the intestine and pyloric caeca, and perivascular inflammation in the liver (Mullins et al., 1998; Rimstad et al., 1999). Clinical signs appear 2-4 weeks after the infection. Studies have shown that some fish may harbor the virus for many weeks before succumbing to ISAV-induced disease and that this may therefore be important in the dissemination of the infection within a farm (Rimstad et al., 1999). At terminal stages of the disease, the gross pathologic changes of the

disease include exophthalmia, leukopenia, ascites, congestion and enlargement of liver and spleen, congestion in the intestinal mucosa, hemorrhagic liver necrosis, and petechiae in the visceral fat (Thorud and Djupvik 1988, Evensen et al., 1991; Hjeltnes et al., 1992; Olsen et al., 1992; Dannevig et al., 1993; Nylund et al., 1994). It has been shown that Atlantic salmon that recovered from a clinical infection can continue to shed virus for long periods of time, but detection of ISAV in carrier fish required the use of RT-PCR (Devold et al., 2000) suggesting that the viral RNA is not abundant. The underlying cellular and molecular mechanisms of pathogenesis and persistence of ISAV in fish is not fully elucidated.

1.2.4.8. Immune response to ISAV infection

Little information is available on immune response to ISAV infection. Such information is important in understanding the pathogenesis of the disease and to develop efficient control strategies. Information from commercial farms indicates that the fish that survive ISAV infection do not have an increased susceptibility to other viral or bacterial infections (Falk et al., 1995), suggesting that ISAV infection did not cause immunosuppression in infected fish. Further experimental infection also showed the resistance of ISA survivors to reinfection (Falk et al., 1995). Protective immune response to ISAV has been demonstrated in experimentally infected fish as well as in vaccination trials (Dannevig et al., 1993; Jones et al., 1999; Brown et al., 2000). An increase in the proliferative response of head kidney leukocytes to T-cell mitogen phytohemagglutinin (PHA) was observed in Atlantic salmon one week post-infection (Dannevig et al., 1993). Later in the course of infection either PHA or lipopolysaccharide (LPS) failed however to elicit any response in the head kidney

leukocytes, indicating an impaired function of these cells (Dannevig et al., 1993). Signs of macrophage activation, and increased number of immunoglobulin-positive cells were also observed in both spleen and kidney in the early stages of the infection (Falk et al., 1995a). The ISA-induced changes in spleen and head kidney may also result from an activation of the immune system, as cells associated with the ellipsoids of the spleen show reactivity for immunoglobulin and complement factor C3, indicating immune complex trapping (Falk et al. 1995a).

The importance of humoral factors in the immune response to ISAV was demonstrated early by passive immunization studies (Falk et al., 1995). Experimental infections demonstrated a protective immune response in Atlantic salmon against ISAV. This protective immune response does not however, provide full protection against the disease, since high mortality has usually been observed in both naturally and experimentally infected fish (Thorud 1991; Dannevig et al., 1993; Falk et al., 1995). In contrast to higher vertebrates, teleost fish have only two immunoglobulin (Ig) heavy chain isotypes: IgM and IgD (Hordvik 1998; Hordvik et al., 1999). It has been suggested that different forms of fish IgM and its observed flexibility of structure may compensate for a lack of immunoglobulin class diversity (Kaattari et al., 1998). Although previous studies reported increased resistance of Atlantic salmon to ISAV upon re-infection or after passive immunization with serum from fish that had recovered from ISA (Falk and Dannevig 1995), or following vaccination with inactivated virus (Brown et al., 2000; Jones et al., 1999b), antibody levels in such fish were not determined. A recently developed ELISA system for detection of ISAV antibody (Kibenge

et al., 2002) showed that farmed fish presented two different types of antibody responses to ISAV. Naturally infected Atlantic salmon carrying ISAV that was detected by RT-PCR had a specific antibody response to ISAV suggestive of a recent infection. Those fish that were virus-negative by RT-PCR had an elevated non-specific antibody reactivity that may be suggestive of chronic infection or resistance to ISAV. Sera from experimental fish collected up to 6 weeks after infection with ISAV did not show the elevated non-specific antibody reactivity (Kibenge et al., 2002). Atlantic salmon antibody response to ISAV studied by Western blot analysis using Atlantic salmon anti-ISAV polyclonal sera showed that antibodies are produced mainly against the 72 kDa NP and 42 kDa haemagglutinin proteins of ISAV, suggesting that these proteins may be important antigens of ISA vaccines (Clouthier et al., 2002).

Monoclonal antibodies are widely used for the antigenic analysis of viral proteins. They provide information about the virus structure and the immunological defense mechanism against virus infection which could not otherwise be obtained (McCullough 1986).The discriminatory power of hybridoma antibodies make them ideal reagents for investigation of antigenic relationships between viral proteins. They can be used to determine what viral antigens are expressed on infected cell surfaces and to characterize the antigenic topology of viral proteins, which is essential for developing sensitive diagnostic tests and formulating efficacious vaccines (Yewdell and Gerhard 1981; McCullough 1986). A monoclonal antibody (IgG1 isotype) designated 3H6F8 raised against ISAV was characterized (Falk et al., 1998). Immunofluorescence staining showed that this mAb was directed against a

cytoplasmic ISAV protein. This mAb showed a strong reaction with IFAT on ISAV infected cell cultures, and with ELISA using lysed-infected cell cultures as antigen, but failed to react with ISAV polypeptides in Western blots, under both reducing and non-reducing conditions. Reaction was also not observed on formalin-fixed paraffin embedded tissue sections from diseased fish (Falk et al., 1998). Monoclonal antibody 3H6F8 has been shown to be haemagglutinin specific based on its neutralizing, heamagglutination inhibition activities, and reactivity to recombinant haemagglutinin expressed in baculovirus (Falk et al., 1998; Krossoy et al., 2001; Rimstad et al., 2001).

1.2.4.9. Diagnosis of ISAV

The laboratory diagnostic assays for ISA include histological examination (Evensen et al., 1991; Falk et al., 1995; Speilberg et al., 1995; Byrne et al., 1998; McClure et al., 2003) and/or electron microscopy (Hovland et al., 1994) of fish tissues, the isolation of ISAV in SHK-1 (Dannevig et al. 1995a,b) and/or CHSE-214 cell lines (Bouchard et al. 1999; Kibenge et al. 2000a) and electron microscopic examination of a positive isolate, the use of RT-PCR (Mjaaland et al., 1997; Rimstad et al., 1999; Blake et al., 1999; Bouchard et al., 1999; Kibenge et al. 2000a, 2001b; Devold et al. 2000; Mikalsen et al., 2001; Lovdal and Enger, 2002), and IFAT on positive virus isolates or on tissue samples from suspected fish (Falk and Dannevig, 1995; Falk et al., 1998; Mjaaland et al., 1997; Blake et al., 1999; Bouchard et al., 1999; Rimstad et al., 1999), and finally *in-situ* hybridization on tissue samples from suspected fish (Gregory, 2002; Moneke et al., 2003).

Fish immunoglobulins possess a similar range of serological activities to those shown by mammalian immunoglobulins (Corbel 1975). In contrast to diagnostic tests for infectious diseases of mammals and birds (Anon. 2000c), confirmation of exposure to a disease agent by detection of antibody to the agent is rarely, if ever, used in aquatic animal disease diagnosis. Present limitations are due not only to difficulties with interpretation of serological test results (Hattenberger-Baudouy et al., 1995) but also to discrete features of the humoral immune response in fish (Corbel 1975, Dorson 1981) such as a predominance of the IgM subtype (Marchalonis 1971) and absence of switch by B-lymphocytes to secretion of high-affinity binding antibodies (Wilson and Warr 1992). Fish serology [for example haemagglutination inhibition (HI), antibody ELISA, and VN] could be of importance for detecting asymptomatic virus carriers among fish stocks for various viral diseases, but is not yet validated, and no antibody detection test is yet approved by regulatory authorities. Thus, antibody detection tests are currently used in fish disease diagnosis only to supplement other diagnostic methods such as virus isolation by cell culture or RT-PCR (Kibenge et al., 2002). There remains a need for diagnostic approaches, other than direct detection of the virus, for the assessment of fish populations relevant to infection with virus pathogens.

Recently, an indirect ELISA and indirect competitive ELISA that could detect ISAV-specific antibodies in the sera of infected and/or vaccinated fish have been described (Kibenge et al., 2002). In addition, a one-tube real-time RT-PCR using LightCycler technology (Roche Applied Science) and SYBR Green chemistry that quantitatively detects ISAV in biological samples has been developed (Munir and Kibenge 2004). The result can be obtained in only

80 minutes. The detection limit of the assay was 0.006 ng of ISAV RNA. This was 100 times more sensitive than the conventional one-tube RT-PCR assay. The higher analytical sensitivity of real-time RT-PCR is in part due to the computerized real-time detection of the amplification, since the detection of PCR products by gel electrophoresis shows no difference in sensitivity between real-time RT-PCR and conventional RT-PCR (Munir and Kibenge 2004). The real-time RT-PCR technique also has the potential to quantitate virus that replicates without production of CPE (i.e., noncytopathic virus) in cell cultures. None of these methods has yet been validated by reference authorities, such as the National Veterinary Services Laboratories in Ames, Iowa, USA (Merrill, 2003). According to the OIE, the presence of ISA is officially confirmed if any one of the following three criteria has been met: (i) clinical signs and postmortem findings of ISA together with detection of ISAV by cell culture, IFAT, or RT-PCR; (ii) isolation and identification of ISAV in two samples on separate occasions; and (iii) isolation and identification of ISAV from at least one sample with corroborating evidence of ISAV in tissue preparations from any fish on the farm by IFAT or PCR (Hastein 2003).

1.2.4.10. Control of ISAV

Various eradication programs have been established to control ISAV outbreaks (Mullins 1998). It has been shown that virus is readily transmitted in sea water. Such dissemination increases the risk of contamination of culture facilities within 5-6 km of an infected site under conditions found in Norway (Jarp and Karlsen 1997). It is, therefore, recommended that culture sites must be spaced sufficiently apart and that wastewater from slaughter and

processing facilities should be thoroughly disinfected (Jarp and Karlsen 1997). Further contagion may be managed by control of ship and personnel movements among sites, destruction of infected fish groups, and fallowing of contaminated sites. Iodophore, chloramine-T, and chlorine dioxide have been shown to be effective topical disinfectant against ISAV (Cipriano 2002).

Vaccination against ISAV is considered to be a very important strategy in controlling the disease by protecting vulnerable stocks followed by eradication strategies. Increased survival of fresh-water-reared Atlantic salmon vaccinated with inactivated whole ISAV emulsified with mineral oil and then experimentally injected with ISAV has been reported (Jones et al., 1999; Brown et al., 2000). This is the basis for current ISAV vaccines used in Canada. It has been shown however that the immunity engendered by these vaccines is neither 100 percent protective nor sterile (Kibenge et al., 2003). Currently, ISAV vaccination is only permitted in North America, but could also be used in Norway under specific conditions. Vaccine companies have, therefore, applied for permission to conduct clinical trials in Europe in accordance with ISAV contingency plans (Salonius et al., 2003). Sequence analysis of the ISAV haemagglutinin gene and cross-neutralization studies suggest that there is significant antigenic variation among ISAV isolates, and that ISAV vaccines would need to induce broadly neutralizing antibodies in order to protect under field conditions.

1.3. Hypotheses and research objectives

ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon (Nylund et al., 1994, 1996; Dannevig et al., 1995a,b; Byrne et al., 1998; Rodger et al., 1998) and asymptomatic infection in feral fish (Raynard et al., 2001a). The immune response following ISAV infection does not provide full protection against the disease, since high mortality has usually been observed in both naturally and experimentally infected fish (Thorud 1991; Dannevig et al., 1993; Falk et al., 1995) and Atlantic salmon that recovered from a clinical infection can continue to shed virus for long periods of time. But detection of ISAV in carrier fish required the use of RT-PCR (Devold et al., 2000) suggesting that the viral RNA is not abundant. Information is lacking on the host-pathogen interactions of this virus which is essential in understanding the mechanisms of pathogenesis and persistence of ISAV.

In Atlantic salmon, ISAV seems to target leucocytic cells (Falk et al., 1995) and endothelial cells (Falk & Dannevig 1995) or endothelial-associated cells (Falk et al., 2001) and macrophages (Moneke et al., 2003). *In-vitro* ISAV readily replicates in macrophage-like cell lines such as SHK-1, TO, and ASK-2 which are derived from the Atlantic salmon pronephros (Dannevig et al., 1995; Wergeland & Jakobsen 2001; Rolland et al., 2002). Some strains of ISAV can also replicate and cause CPE in CHSE -214 cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001). These cell lines are distinctly different in their growth characteristics and they display characteristic viral-induced CPE (Kibenge et al., 2001; Rolland et al., 2002). The mechanisms of cell death and CPE caused by ISAV

infection in cultured cells have not previously been studied. Information on how this virus interacts with the host cell will provide a better understanding of the determinants of viral pathogenesis in its natural hosts.

Currently there is little basic information on how this virus interacts with the host defenses, which is an issue to be considered when planning potential vaccination strategies. Preliminary VN studies on ISAV using rabbit and fish polyclonal antisera to ISAV suggested an antibody-mediated internalization of the virus in SHK-1 and TO, which are macrophage-like cell lines (Dr. Kibenge pers.comm). Total virus neutralization was observed in CHSE-214 cells, whereas both rabbit and fish sera neutralized the virus to a lesser degree in SHK-1 and TO cells. Therefore, this thesis research was based on two hypotheses, (a) that ISAV persists in fish by causing a low level noncytocidal infection of macrophage-like cells and by inhibiting host antiviral responses; and (b) that fish macrophages are able to effectively internalize antibody-coated virus particles through Fc receptors as do mammalian macrophages, thereby allowing cellular penetration of antibody-coated ISAV and subsequent virus replication. A similar type of antibody-enhanced ISAV infection of macrophages could occur *in-vivo* in the presence of sub-neutralizing antibodies. Such a non-beneficial and paradoxical infection via Fc receptors would further contribute to the pathogenicity and persistence of ISAV in fish. The primary goal of this thesis research was to investigate host-pathogen interactions of ISAV. Specific objectives of this study were to:

1. Investigate the mechanisms of cell death during ISAV-infection of fish cell lines

2. Investigate antibody-mediated uptake and replication of ISAV by macrophage-like fish cell lines

3. Characterize selected monoclonal antibodies to ISAV

1.4. References

1. ADAMS, J. M., CORY, S.: The Bcl-2 protein family: arbiters of cell survival. Science 1998; 281: 1322-1326

2. ALONSO-CAPLEN, F. V., NEMEROFF, M. E., QIU, Y., KRUG, R. M.: Nucleocytoplasmic transport: the influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. Genes Dev. 1992; 6: 255-267

3. AMEISEN, J.C., CAPRON, A: Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol Today. 1991; 12: 102-105

4. ANONYMOUS. Infectious salmon anaemia in the Faroe Islands. 13, 53-59. 2000. Disease Information, Office International des Epizooties.

5. ANONYMOUS Assessment of Zoonotic Risk from Infectious Salmon Anaemia virus. Scientific Committee on Animal Health and Animal Welfare Sanco/C3/AH/R18/2000. 2000.

6.ANONYMOUS. Salmon virus detected in Clew Bay fish farm. 2002. The Irish Times, www.ireland.com, August 12, 2002.

7. ANONYMOUS OIE: Manual of standards for diagnostic tests and vaccines, 4th edn. The Office International des Epizooties (OIE), Paris 2000.

8. ARMSTRONG, S. J., OUTLAW, M. C., DIMMOCK, N. J.: Morphological studies of the neutralization of influenza virus by IgM. J. Gen. Virol. 1990; 71: 2313-2319

9. ARMSTRONG, S. J., DIMMOCK, N. J.: Neutralization of influenza virus by low concentrations of hemagglutinin-specific polymeric immunoglobulin A inhibits viral fusion activity, but activation of the ribonucleoprotein is also inhibited. J. Virol. 1992; 66:3823-3832

10. ARMSTRONG, S. J., MCINERNEY, T. L., MCLAIN, L., WAHREN, B., HINKULA,

J., LEVI, M., DIMMOCK, N. J.: Two neutralizing anti-V3 monoclonal antibodies act by affecting different functions of human immunodeficiency virus type 1. J. Gen. Virol.1996; 77: 2931-2941

11. BALACHANDRAN, S., ROBERTS, P.C., KIPPERMAN, T., BHALLA, K.N., COMPANS, R.W., ARCHER, D.R., BARBER, G.N. α/β interferons potentiate virus-induced apoptosis through activation of the FADD/caspase-8 death signaling pathway. J. Virol. 2000; 74: 1513-1523

12. BARBER, G. N.: Host defense, viruses and apoptosis. Cell Death. Differ. 2001; 8: 113-126.

13. BARRY, M., MCFADDEN, G.: Apoptosis regulators from DNA viruses. Curr. Opin. Immunol. 1998; 10: 422-430

14. BIERING, E., FALK, K., HOEL, E., THEVARAJAN, J., JOERINK, M., NYLUND, A., ENDRESEN, C., KROSSOY, B.: Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the colinear transcript from Segment 7 probably encodes a non-structural or minor structural protein. Dis Aquat Org. 2002; 49: 117-122

15. BLAKE, S., BOUCHARD, D., KELEHER, W., OPITZ, M., NICHOLSON, B. L.: Genomic relationships of the North American isolate of infectious salmon anemia virus (ISAV) to the Norwegian strain of ISAV. Dis Aquat Org. 1999; 35: 139-144

16. BLANCOU, J., ANDRAL, B., ANDRAL, L.: A model in mice for the study of the early death phenomenon after vaccination and challenge with rabies virus. J. Gen. Virol.1980; 50; 433-435

17. BOLS, N. C., GANASSIN, R. C., TOM, D. J., LEE, L. E.: Growth of fish cell lines in glutamine-free media. Cytotechnology 1994; 16: 159-166

18. BORSOS T: Complement fixation test. In: *Encyclopedia of immunology* San Diego, California, USA, Acadenic Press, 1992, pp. 381-382.

19. BOUCHAR D., BROCKWAY, K., GIRAY, C., KELEHER, W., MERRIL, P. L: First report of infectious salmon anemia (ISA) in the United States. Bull. Eur. Ass. Fish. Pathol 2001; 21: 86-88

20. BOUCHARD, D., KELEHER, W., OPITZ, H. M., BLAKE, S., EDWARDS, K. C., NICHOLSON, B. L.: Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Dis Aquat Org.1999; 35:131-137

21. BOYER, V., DESGRANGES, C., TRABAUD, M. A., FISCHER, E., KAZATCHKINE,

M. D.: Complement mediates human immunodeficiency virus type 1 infection of a human T cell line in a CD4- and antibody-independent fashion. J. Exp. Med. 1991; 173: 1151-1158

22. BOYER, V., DELIBRIAS, C., NORAZ, N., FISCHER, E., KAZATCHKINE, M. D., DESGRANGES, C.: Complement receptor type 2 mediates infection of the human CD4-negative Raji B-cell line with opsonized HIV. Scand. J. Immunol. 1992; 36: 879-883

23. BRECKENRIDGE, D. G., GERMAIN, M., MATHAI, J. P., NGUYEN, M., SHORE, G. C.: Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 2003; 22: 8608-8618

24. BRICKNEL, L.I.R., BRUNO, D.W., CUNNIGHAM, C., HASTINGS, T.S., McVICAR, A.H., MUNRO, P. D., RAYNARD, R., STAGG, R.M. Report on the first occurrence of infectious salmon anemia (ISA) in Atlantic salmon in Scotland, United Kingdom. Proceedings of the Third International Symposium on Aquatic Animal Health (Baltimore, MD 30, August-3 September 1998).

25. BROWN, L.L., SPERKER, S.A., CLOUTHIER, S., THORNTON, J.C: Development of a vaccine against infectious salmon anaemia virus (ISAV). 100: 4-7. Bull. Aquat. Ass. Canada 2000; 100: 4-7

26. BROWN, E. G.: Influenza virus genetics. Biomedicine & Pharmacotherapy 2000; 54: 196-209

27. BULLOUGH, P. A., HUGHSON, F. M., SKEHEL, J. J., WILEY, D. C.: Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 1994; 371: 37-43

28. BURSTIN, S. J., BRANDRISS, M. W., SCHLESINGER, J. J.: Infection of a macrophage-like cell line, P388D1 with reovirus; effects of immune ascitic fluids and monoclonal antibodies on neutralization and on enhancement of viral growth. J. Immunol.1983; 130: 2915-2919

29. BURTON, D. R., SAPHIRE, E. O., PARREN, P. W.: A model for neutralization of viruses based on antibody coating of the virion surface. Curr. Top. Microbiol. Immunol. 2001; 260: 109-143

30. BURTON, D. R.: Antibodies, viruses and vaccines. Nat. Rev. Immunol. 2002; 2: 706-713

31. BUTTKE, T. M., SANDSTROM, P. A.: Oxidative stress as a mediator of apoptosis. Immunol. Today 1994; 15; 7-10

32. BYRNE, P. J., MAC PHEE D. D., OSTLAND, V. E., JOHNSON, G., FERGUSON, H.

W: Haemorrhagic kidney syndrome of Atlantic salmon. Journal of Fish Diseases 1998; 21: 81-91

33. CARDOSA, M. J., PORTERFIELD, J. S., GORDON, S.: Complement receptor mediates enhanced flavivirus replication in macrophages. J. Exp. Med. 1983; 158: 258-263

34. CARRASCO, L., SMITH, A. E.: Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. Nature 1976; 264: 807-809

35. CECILIA, D., GHOSH, S. N.: Antibody dependent plaque enhancement by monoclonal antibodies against Japanese encephalitis virus. Indian J. Med. Res. 1988; 87: 521-525

36. CENTRELLA, M., LUCAS-LENARD, J.: Regulation of protein synthesis in vesicular stomatitis virus-infected mouse L-929 cells by decreased protein synthesis initiation factor 2 activity. J. Virol. 1982; 41: 781-791

37. CHANOCK, R. M., KAPIKIAN, A. Z., MILLS, J., KIM, H. W., PARROTT, R. H.: Influence of immunological factors in respiratory syncytial virus disease. Arch. Environ. Health 1970; 21: 347-355

38. CHEN, Z., LI, Y., KRUG, R. M.: Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. EMBO J. 1999; 18: 2273-2283

39. CHEN, W., CALVO, P.A., MALID, D., GIBBS, J., SCHUBERT, U., BACIK, I., BASTA, S., O'Neill, SCHICKLI, J., PALESE, P., HENKLEI, P., BENNINK, J.R., YEWDELL, J.W. A novel influenza virus mitochondrial protein that induces cell death. Nature Medicine. 2001; 7: 1306-1312

40. CIPRIANO, R. C. Infectious salmon anaemia virus. Fish disease leaflet. United States Geographical Survey, National fish health research laboratory, 11700 Leetown Road, Kearneysville, WV 25430, USA. 2002; 85: 1-11

41. CLOUTHIER, S. C., RECTOR, T., BROWN, N. E., ANDERSON, E. D.: Genomic organization of infectious salmon anaemia virus. J. Gen. Virol.2002; 83: 421-428

42. COOK, M., VINCENT, S., RITCHIE, R., GRIFFITHS, S.: Development of a strain typing assay for infectious salmon anemia virus (ISAV). In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries. 2003; pp 75-86

43. COOK, M., GRIFFITHS, S., CUSACK, R., McGEACHY, S., AND RITCHIE, R: Identification and characterization of infectious salmon anaemia virus (ISAV) haemagglutinin gene highly polymorphic region (HPR) type 0 in North America. Bull. Eur. Ass. Fish Pathol. 2004; (In Press)

44. CORAPI, W. V., OLSEN, C. W., SCOTT, F. W.: Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. J. Virol. 1992; 66: 6695-6705

45. CORBEL MJ: The immune response in fish: a review. J. Fish Biol 1975; 7: 539- 563

46. COTRAN RS, KUMAR V, COLLINS T: Cell injury and cell death. In: *Robbins Pathologic Basis of Disease* Philadelphia, USA, W B Sounders company, 1999, pp. 1-28.

47. COX, N.J., FULLER. F., KAVERIN. N., KLENK. H.D., LAMB. R.A., MAHY, B.W.J., MCCAULEY, J., NAKAMURA, K., PALESE. P., WEBSTE,. R: Orthomyxoviridae. In: Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses((M.H.V. van Regenmortel, C. M. Faquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, R. B. Wickner, eds Springer-Verlag, Wien /New York, 2002, pp. 585-597.

48. CUNNINGHAM, C. O., SNOW, M.: The genetics of infectious salmon anemia virus. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp 87-95

49. CUNNINGHAM, C. O., SNOW, M.: Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. Dis Aquat Org 2000; 41: 1-8

50. DANNEVIG BH, FALK K: Atlantic salmon (*Salmo salar* L.) develop infectious salmon anaemia (ISA) after inoculation with in vitro infected leukocytes. J Fish Dis 1994; 17: 183-187

51. DANNEVIG, B.H., BRUDESETH, B.E., GJOEN, T., RODE, M., WERGELAND, H.I., EVENSEN, O., PRESS, C.M..: Characterization of a long- term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 1997; 7:213-226

52. DANNEVIG, B. H., FALK.K, KROSGUD J: Leucocytes from Atlantic Salmon (*Salmo salar)* experimentally infected with ISA exhibit an impaired response to mitogens. J Fish Dis.

1993; 16: 351-359

53. DANNEVIG, B. H., FALK, K., NAMORK, E.: Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol 1995a; 76: 1353-1359

54. DANNEVIG, B. H., FALK, K., PRESS, C. M.: Propagation of infectious salmon anaemia (ISA) virus in cell culture. Vet Res 1995b; 26: 438-442

55. DAVIES, M. V., CHANG, H. W., JACOBS, B. L., KAUFMAN, R. J.: The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. J. Virol. 1993; 67: 1688-1692

56. DEGTEREV, A., BOYCE, M., YUAN, J.: A decade of caspases. Oncogene 2003; 22: 8543-8567

57. DEVERAUX, Q.L, TAKAHASHI, R., SALVESEN, G.S., REED, J.C. X-linked IAP is a direct inhibitor of cell-death proteases. Nature. 1997; 388:300-304

58. DEVOLD, M., KROSSOY, B., ASPEHAUG, V., NYLUND, A.: Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. Dis Aquat Org 2000; 40: 9-18

59. DEVOLD, M., FALK, K., DALE, B., KROSSOY, B., BIERING, E., ASPEHAUG, V., NILSEN, F., NYLUND, A.: Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. Dis. Aquat. Org 2001; 47: 119-128

60. DIAZ-GUERRA, M., RIVAS, C., ESTEBAN, M.: Activation of the IFN-inducible enzyme RNase L causes apoptosis of animal cells. Virology 1997; 236: 354-363

61. DIMITROV, T., KRAJCSI, P., HERMISTON, T. W., TOLLEFSON, A. E., HANNINK, M., WOLD, W. S.: Adenovirus E3-10.4K/14.5K protein complex inhibits tumor necrosis factor-induced translocation of cytosolic phospholipase A2 to membranes. J. Virol. 1997; 71: 2830-2837

62. DIMMOCK N.J: Update on the neutralization of animal viruses. Med Virol 1995; 5: 165-179

63. DIMMOCK, N. J.: Neutralization of animal viruses. Curr. Top. Microbiol. Immunol. 1993 183: 1-149

64. DORSON, M: Role and characterization of fish antibodies. In: Hennessen W (ed)

Developments in biological standardization, fish biologics and vaccines, Karger, Basel 1981; 49: 307-319

65. DRATEWKA-KOS, E., KISS, I., LUCAS-LENARD, J., MEHTA, H. B., WOODLEY, C. L., WAHBA, A. J.: Catalytic utilization of eIF-2 and mRNA binding proteins are limiting in lysates from vesicular stomatitis virus infected L cells. Biochemistry 1984; 23: 6184-6190

66. EDWARDS, M. J., DIMMOCK, N. J.: Two influenza A virus-specific Fabs neutralize by inhibiting virus attachment to target cells, while neutralization by their IgGs is complex and occurs simultaneously through fusion inhibition and attachment inhibition. Virology 2000; 278: 423-435

67. EGGER, L., SCHNEIDER, J., RHEME, C., TAPERNOUX, M., HACKI, J., BORNER, C.: Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. Cell Death. Differ. 2003; 10: 1188-1203

68. ELIASSEN, T. M., FROYSTAD, M. K., DANNEVIG, B. H., JANKOWSKA, M., BRECH, A., FALK, K., ROMOREN, K., GJOEN, T.: Initial events in infectious salmon anemia virus infection: evidence for the requirement of a low-pH step. J Virol 2000; 74: 218-227

69. ELIOPOULOS, A. G., BLAKE, S. M., FLOETTMANN, J. E., ROWE, M., YOUNG, L. S.: Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. J. Virol. 1999; 73: 1023-1035

70. EVENSEN, O., THORUD, K. E., OLSEN, Y. A.: A morphological study of the gross and light microscopic lesions of infectious anaemia in Atlantic salmon (*Salmo salar*). Res Vet Sci 1991; 51: 215-222

71. FALK, K., DANNEVIG, B. H., A: Demonstration of a protective immune response in infectious salmon anaemia (ISA) infected Atlantic salmon *Salmo salar*. Dis Aquat Org 1995; 21: 1-5

72. FALK, K., ASPEHAUG, V., VLASAK, R., ENDRESEN, C.: Identification and Characterization of Viral Structural Proteins of Infectious Salmon Anemia Virus. J Virol 2004; 78: 3063-3071

73. FALK, K., DANNEVIG, B. H.: Demonstration of infectious salmon anaemia (ISA) viral antigens in cell cultures and tissue sections. Vet. Res. 1995; 26: 499-504

74. FALK, K., PRESS, C. M., LANDSVERK, T., DANNEVIG, B. H.: Spleen and kidney of Atlantic salmon (*Salmo salar* L.) show histochemical changes early in the course of

experimentally induced infectious salmon anaemia (ISA). Vet Immunol Immunopathol 1995a; 49: 115-126,.

75. FALK, K., NAMORK, E., RIMSTAD, E., MJAALAND, S., DANNEVIG, B. H.: Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). J Virol 1997; 71: 9016-9023

76. FALK, K., NAMORK, E., DANNEVIG, B. H.: Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. Dis Aquat Org 1998; 34: 77-85

77. FANIDI, A., HANCOCK, D. C., LITTLEWOOD, T. D.: Suppression of c-Myc-induced apoptosis by the Epstein-Barr virus gene product BHRF1. J. Virol. 1998; 72: 8392-8395

78. FEIGENBLUM, D., SCHNEIDER, R. J.: Modification of eukaryotic initiation factor 4F during infection by influenza virus. J. Virol. 1993; 67: 3027-3035

79. FERNANDEZ-TOMAS, C.: The presence of viral-induced proteins in nuclei from poliovirus-infected HeLa cells. Virology 1982; 116: 629-634

80. FERRAN, M. C., LUCAS-LENARD, J. M.: The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. J. Virol. 1997; 71: 371-377

81. FLINT S J, ENQUIST L W, KRUG R M, RACANIELLO V R, SKALKA A M: Virus cultivation, detection, and genetics. In: *Principles of Virology* Washinton, DC, American Society for Microbiology, 2000, pp. 33-37.

82. FORTES, P., BELOSO, A., ORTIN, J.: Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. EMBO J. 1994; 13: 704-712

83. FRIBORG, J., Jr., KONG, W., HOTTIGER, M. O., NABEL, G. J.: p53 inhibition by the LANA protein of KSHV protects against cell death. Nature 1999; 402: 889-894

84. FRY, E. E., LEA, S. M., JACKSON, T., NEWMAN, J. W., ELLARD, F. M., BLAKEMORE, W. E., ABU-GHAZALEH, R., SAMUEL, A., KING, A. M., STUART, D. I.: The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. EMBO J. 1999; 18: 543-554

85. FUST, G.: Enhancing antibodies in HIV infection. Parasitology 1997, 115 Suppl, S:127-S140

86. GIMENEZ, B.: Antibody-mediated enhancement of respiratory syncytial virus infection. J. Infect. Dis.1990: 162: 1211

87. GIRN, J., KAVOOSI, M., CHANTLER, J.: Enhancement of coxsackievirus B3 infection by antibody to a different coxsackievirus strain. J. Gen. Virol. 2002; 83: 351-358

88. GREEN, D. R., REED, J. C.: Mitochondria and apoptosis. Science 1998; 281: 1309-1312

89. GREGORY A: Detection of infectious salmon anaemia virus (ISAV) by in situ hybridization. Dis Aquat Org 2002; 50: 105-110

90. GRIFFITHS, S., COOK. M., MALLORY, B., RITCHIE, R.: Characterization of ISAV proteins from cell culture. Dis Aquat Org 2001; 45: 19-24

91. HALSTEAD, S. B., SCANLON, J. E., UMPAIVIT, P., UDOMSAKDI, S.: Dengue and chikungunya virus infection in man in Thailand, 1962-1964. IV. Epidemiologic studies in the Bangkok metropolitan area. Am. J. Trop. Med. Hyg. 1969; 18: 997-1021

92. HALSTEAD, S. B., NIMMANNITYA, S., COHEN, S. N.: Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J. Biol. Med. 1970; 42: 311-328

93. HALSTEAD, S. B.: Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion. Yale J. Biol. Med. 1970; 42: 350-362

94. HALSTEAD, S. B., O'ROURKE, E. J.: Antibody-enhanced dengue virus infection in primate leukocytes. Nature 1977; 265: 739-741

95. HALSTEAD, S. B., O'ROURKE, E. J.: Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 1977; 146: 201-217

96. HALSTEAD, S. B.: In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J. Infect. Dis. 1979; 140: 527-533

97. HALSTEAD, S. B.: Immune enhancement of viral infection. Prog. Allergy 1982; 31: 301-364

98. HALSTEAD, S. B., VENKATESHAN, C. N., GENTRY, M. K., LARSEN, L. K.: Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. J. Immunol. 1984; 132: 1529-1532

99. HAN, J., SABBATINI, P., PEREZ, D., RAO, L., MODHA, D., WHITE, E.: The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev. 1996; 10: 461-477

100. HARDWICK, J. M., GRIFFIN, D. E.: Viral effects on cellular functions. In Viral

pathogenesis. In: NATHANSON, N., ET AL, eds., *Viral pathogenesis* Lippincott-Raven Publishers, Philadelphia, USA, 1997, pp. 55-79.

101. HASTINGS ,T., OLIVIER, G., CUSACK, R., BRICKNELL, I., NYLUND, A.: Infectious salmon anaemia. Bull Euro Ass Fish Pathol1999; 19: 286-288

102. HATTENBERGER-BAUDONY, A.M., DANTON, M., MERLE, G. D. K..: Serum neutralization test for epidemiological studies of salmonid rhabdovirus in France. Vet Res 1995; 26: 512-520

103. HAWKES R, A.: Enhancement of infectivity of arboviruses by specific antisera produced in domestic fowls. Aust J Exp Biol Med Sci 1964; 42: 465-482

104. HAWKES, R. A., LAFFERTY, K. J.: The enchancement of virus infectivity by antibody. Virology 1967; 33: 250-261

105. HA,Y., S., KANNOURAKIS, G.: A time to kill: viral manipulation of the cell death program. J. Gen. Virol. 2002; 83: 1547-1564

106. HELLEBO, A., VILAS, U/, FALK, K., VLASAK, R: Infectious salmon anaemia virus specifically binds to and hydrolyzes 4-O-acetylated sialic acids. J Virol 2004; 78: 3055-3062

107. HENCHAL, E. A., MCCOWN, J. M., BURKE, D. S., SEGUIN, M. C., BRANDT, W. E.: Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. Am. J. Trop. Med. Hyg. 1985; 34: 162-169

108. HER, L. S., LUND, E., DAHLBERG, J. E.: Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. Science 1997; 276: 1845-1848

109. HJELTNES, B., SAMUELSEN, O.B., SVARDAL: Changes in plasma and liver glutathione levels in Atlantic salmon suffering from ISA. Dis Aqua Org 1992; 14: 31-37

110. HOHDATSU, T., NAKAMURA, M., ISHIZUKA, Y., YAMADA, H., KOYAMA, H.: A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. Arch. Virol. 1991; 120: 207-217.

111. HOHDATSU, T., TOKUNAGA, J., KOYAMA, H.: The role of IgG subclass of mouse monoclonal antibodies in antibody-dependent enhancement of feline infectious peritonitis virus infection of feline macrophages. Arch. Virol. 1994; 139: 273-285

112. HOMSY, J., MEYER, M., TATENO, M., CLARKSON, S., LEVY, J. A.: The Fc and

not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science 1989; 244: 1357-1360

113. HORDVIK. I.: The impact of ancestral tetraploidy on antibody heterogeneity in salmonid fishes. Immunol Rev 1998; 166: 153-157

114. HORDVIK, I., THEVARAJAN, J., SAMDAL, I., BASTANI, N., KROSSOY, K.: Molecular cloning and phylogenic analysis of the Atlantic salmon immunoglobulin D gene. Scand J Immunol 1999; 50: 202-210

115. HOVLAND, T., NYLUND, K., WATANABE, ENDRESEN, C: Observation of infectious salmon anemia virus in Atlantic salmon, *Salmo salar* L. J Fish Dis. 1994; 17: 291-296

116. INADA, T., CHONG, K. T., MIMS, C. A.: Enhancing antibodies, macrophages and virulence in mouse cytomegalovirus infection. J. Gen. Virol. 1985; 66: 871-878

117. INGLIS, J. A., BRUCE, J., CUNNINGHAM, C. O.: Nucleotide sequence variation in isolates of infectious salmon anaemia virus (ISAV) from Atlantic salmon *Salmo salar* in Scotland and Norway. Dis Aquat Org 2000; 43: 71-76

118. IORIO, R. M., GLICKMAN, R. L., RIEL, A. M., SHEEHAN, J. P., BRATT, M. A.: Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. Virus Res. 1989; 13: 245-261

119. ISSEL, C. J., HOROHOV, D. W., LEA, D. F., ADAMS, W. V., Jr., HAGIUS, S. D., MCMANUS, J. M., ALLISON, A. C., MONTELARO, R. C.: Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. J. Virol. 1992; 66: 3398-3408

120. ITOH, N., YONEHARA, S., ISHII, A., YONEHARA, M., MIZUSHIMA, S., SAMESHIMA, M., HASE, A., SETO, Y., NAGATA, S.: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991; 66: 233-243

121. IWASAKI, M., INOUE, S., TROY, F. A.: A new sialic acid analogue, 9-O-acetyl-deaminated neuraminic acid, and alpha -2,8-linked O-acetylated poly(N-glycolylneuraminyl) chains in a novel polysialoglycoprotein from salmon eggs. J. Biol. Chem.1990; 265: 2596-2602

122. JARP, J., KARLSEN, E.: Infectious salmon anemia risk factors in sea-cultured Atlantic salmon Salomo salar. Dis Aqua Org 1997; 28: 79-86

123. JOLLY, P. E., HUSO, D., HART, G., NARAYAN, O.: Modulation of lentivirus

replication by antibodies. Non-neutralizing antibodies to caprine arthritis-encephalitis virus enhance early stages of infection in macrophages, but do not cause increased production of virions. J Gen Virol 1989; 70: 2221-2226

124. JONES, S. R. M., MACKINNON, M.A., GROMAN, B.D: Virulence and pathology of infectious salmon anaemia virus isolated from Farmed salmon in Atlantic Can J Aquat An Health 1999; 11: 400-405

125. JORDAN, R., WANG, L., GRACZYK, T. M., BLOCK, T. M., ROMANO, P. R.: Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J. Virol. 2002; 76: 9588-9599

126. JOUAULT, T., CHAPUIS, F., BAHRAOUI, E., GLUCKMAN, J. C.: Infection of monocytic cells by HIV1: combined role of FcR and CD4. Res. Virol. 1991; 142: 183-188

127. JUNG, Y. K., YUAN, J.: Suppression of interleukin-1beta converting enzyme (ICE)-induced apoptosis by SV40 large T antigen. Oncogene 1997; 14: 1207-1214

128. KAATTARI, S., EVANS, D., KLEMER, J.: Varied redox forms of teleost IgM: an alternative to isotypic diversity? Immunol. Rev. 1998; 166: 133-142

129. KANNO, H., WOLFINBARGER, J.B., BLOOM, M.E. Aleutian mink disease parvovirus infection of mink macrophages and human macrophage cell line U937: Demonstration of antibody-dependent enhancement of infection. J Virol 67: 7017-7024

130. KAPIKIAN, A. Z., MITCHELL, R. H., CHANOCK, R. M., SHVEDOFF, R. A., STEWART, C. E.: An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. 1969; 89: 405-421

131. KAWAOKA, Y., NESTOROWICZ, A., ALEXANDER, D. J., WEBSTER, R. G.: Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. Virology 1987; 158: 218-227

132. KENDAL, A. P.: A comparison of "influenza C" with prototype myxoviruses: receptor-destroycing activity (neuraminidase) and structural polypeptides. Virology 1975; 65: 87-99

133. KEDAR, E., ORTIZ DE LANDAZURI, M., FAHEY, J.L. Comparative studies of immunoglobulin receptors and antibody-dependent cell cytotoxicity (ADCC) in rat lymphoid organs. J. Immunol. 1974; 112: 37-46

134. KIBENGE, F.S.B., GARATE, O.N., JOHNSON, G., ARRIAGADA, R., KIBENGE,

M.J.T., WADOWSKA, D.: Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Dis Aquat Org 2001a; 45: 9-18

135. KIBENGE, F .S .B, KIBENGE, M.J.T, MCKENNA, P.K., STOTHARD, P., MARSHAL, R., CUSACK, R.R., MCGEACHY, S: Antigenic variation of isolates of infectious salmon anaemia virus correlates with the genetic variation of the viral heamagglutinin gene. J Gen Virol. 2001b; 82: 2869-2879

136. KIBENGE, F. S. B., MUNIR, K., KIBENGE, M. T., JOSEPH, MONEKE. E. Infectious salmon anaemia virus: the causative agent, pathogenesis, and immunity. An Health Res Rev. 2004; In press

137. KIBENGE, F.S.B, KIBENGE, M.T, JOSEPH, T, McDOUGALL, J.: The development of vaccines in Canada in regards to ISAV. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp 39-49

138. KIBENGE, M.J.T, OPAZO, B., ROJAS, A., KIBENGE, F.S.B: Serological evidence of infectious salmon anaemia virus (ISAV) infection in farmed fishes, using an indirect enzyme- linked immunosorbent assay. Dis Aquat Org 2002; 51: 1-11

139. KIBENGE, F. S., LYAKU, J. R., RAINNIE, D., HAMMELL, K. L.: Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. J Gen Virol 2000a; 81: 43-150

140. KIBENGE, F. S., WHYTE, S. K., HAMMELL, K. L., RAINNIE, D., KIBENGE, M. T., MARTIN, C. K.: A dual infection of infectious salmon anaemia (ISA) virus and a togavirus-like virus in ISA of Atlantic salmon *Salmo salar* in New Brunswick, Canada. Dis Aquat Org 2000b; 42: 11-15

141. KIM, H. W., CANCHOLA, J. G., BRANDT, C. D., PYLES, G., CHANOCK, R. M., JENSEN, K., PARROTT, R. H.: Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 1969; 89: 422-434

142. KJELLEN, L., PEREIRA, H.G.: Role of adenovirus anitgens in the induction of neutralizing antibody. J Gen Virol 1968; 2: 177-185

143. KLASSE, P. J., SATTENTAU, Q. J.: Occupancy and mechanism in antibody-mediated neutralization of animal viruses. J. Gen. Virol. 2002; 83: 2091-2108

144. KLIKS, S. C., HALSTEAD, S. B.: Role of antibodies and host cells in plaque enhancement of Murray Valley encephalitis virus. J. Virol. 1983; 46: 394-404

145. KNOSSOW, M., GAUDIER, M., DOUGLAS, A., BARRERE, B., BIZEBARD, T., BARBEY, C., GIGANT, B., SKEHEL, J. J.: Mechanism of neutralization of influenza virus infectivity by antibodies. Virology 2002; 302: 294-298

146. KRAJCSI, P., WOLD, W. S.: Viral proteins that regulate cellular signalling. J. Gen. Virol. 1998; 79: 1323-1335

147. KRISTIANSEN, M., FROYSTAD, M. K., RISHOVD, A. L., GJOEN, T.: Characterization of the receptor-destroying enzyme activity from infectious salmon anaemia virus. J. Gen. Virol. 2002; 83: 2693-2697

148. KROSSOY B, DEVOLD, M, SANDERS, L, PER MORTEN KNAPPSKOG: Cloning and identification of infectious salmon anemia virus haemagglutinin. J Gen Virol 2001a; 82: 1-9

149. KROSSOY, B., HORDVIK, I., NILSEN, F., NYLUND, A., ENDRESEN, C.: The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. J Virol 1999; 73: 2136-2142

150. KROSSOY, B., NILSEN, F., FALK, K., ENDRESEN, C., NYLUND, A.: Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. Dis Aquat Org 2001b; 44: 1-6

151. KUROKAWA, M., KOYAMA, A. H., YASUOKA, S., ADACHI, A.: Influenza virus overcomes apoptosis by rapid multiplication. Int. J. Mol. Med. 1999; 3: 527-530

152. KYUWA, S., COHEN, M., NELSON, G., TAHARA, S. M., STOHLMAN, S. A.: Modulation of cellular macromolecular synthesis by coronavirus: implication for pathogenesis. J. Virol. 1994; 68: 6815-6819

153. LAVOIE, J. N., NGUYEN, M., MARCELLUS, R. C., BRANTON, P. E., SHORE, G. C.: E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. J. Cell Biol. 1998; 140: 637-645

154. LEWIS, J., WESSELINGH, S. L., GRIFFIN, D. E., HARDWICK, J. M.: Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. J. Virol.1996; 70: 1828-1835

155. LEWIS, R. M., COSGRIFF, T. M., GRIFFIN, B. Y., RHODERICK, J., JAHRLING, P. B.: Immune serum increases arenavirus replication in monocytes. J. Gen. Virol. 1988; 69:

1735-1739

156. LI, P., NIJHAWAN, D., BUDIHARDJO, I., SRINIVASULA, S. M., AHMAD, M., ALNEMRI, E. S., WANG, X.: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91: 479-489

157. LI, Q., YAFAL, A. G., LEE, Y. M., HOGLE, J., CHOW, M.: Poliovirus neutralization by antibodies to internal epitopes of VP4 and VP1 results from reversible exposure of these sequences at physiological temperature. J. Virol. 1994; 68: 3965-3970

158. LIDBURY, B. A., MAHALINGAM, S.: Specific ablation of antiviral gene expression in macrophages by antibody-dependent enhancement of Ross River virus infection. J. Virol. 2000; 74: 8376-8381

159. LOVDAL T, ENGER O: Detection of infectious salmon anaemia virus in seawater by nested RT-PCR. Dis Aquat Org 2002; 49: 123-128

160. LOWE, S. W., RULEY, H. E.: Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. 1993; 7: 535-545

161. LU, Y., QIAN, X. Y., KRUG, R. M.: The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes Dev. 1994; 8: 1817-1828

162. LU, Y., WAMBACH, M., KATZE, M. G., KRUG, R. M.: Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. Virology 1995; 214: 222-228

163. LUDWIG, S., PLESCHKA, S., WOLFF, T.: A fatal relationship - Influenza virus interactions with the host cell. Viral Immunology.1999; 12: 175-196

164. LUO, X., BUDIHARDJO, I., ZOU, H., SLAUGHTER, C., WANG, X.: Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. 1998; Cell 94: 481-490

165. LYLES, D. S., MCKENZIE, M. O., AHMED, M., WOOLWINE, S. C.: Potency of wild-type and temperature-sensitive vesicular stomatitis virus matrix protein in the inhibition of host-directed gene expression. Virology 1996; 225: 172-180

166. LYLES, D. S.: Cytopathogenesis and inhibition of host gene expression by RNA viruses. Microbiol. Mol. Biol. Rev. 2000; 64: 709-724

167. LYNGOY. Infectious salmon anaemia in Norway and the Faroe Islands: An industrial approach. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to

infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp 97-109.

168. MaCLEAN, S. A, BOUCHARD, D. A, ELLIS, S. K.: Survey of nonsalmonid marine fishes for detection of infectious salmon anaemia virus and other salmonid pathogens. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp135-143

169. MAIANSKI, N. A., ROOS, D., KUIJPERS, T. W.: Tumor necrosis factor alpha induces a caspase-independent death pathway in human neutrophils. Blood 2003; 101: 1987-1995

170. MARCHALONIS, J.J.: Isolation and partial characterization of immunoglobulins of gold fish (*Carassius auratus*) and carp (*Cyprinus carpio*). Immunology 1971; 20: 161-173

171. MARSHALL, R.R. M.Sc thesis. Comparison of the haemagglutinin genotype with the antigenic type of infectious salmon anaemia virus. Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada. 2003

172. MARSHALL, W. L., YIM, C., GUSTAFSON, E., GRAF, T., SAGE, D. R., HANIFY, K., WILLIAMS, L., FINGEROTH, J., FINBERG, R. W.: Epstein-Barr virus encodes a novel homolog of the bcl-2 oncogene that inhibits apoptosis and associates with Bax and Bak. J. Virol. 1999; 73: 5181-5185

173. McALLISTER, P.E., DENSMORE, C.L., BARBASH, P.A.: Infectious salmon anaemia virus: Injection challenge and waterborne transmission monitored by hematology and polymerase chain reaction assay. 28th Annual Eastern Fish Health Workshop 2003.

174. McCLURE, C.A, HAMMELL, K.L, DOHOO, I.R, STRYHN, H, HAWKINS L.J. Evaluation of infectious salmon anaemia diagnostic tests. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp69-73

175. McCULLOUGH, K. C.: Monoclonal antibodies: implications for virology. Brief review.

Arch. Virol.1986; 87: 1-36

176. McGEACHY, S.M., AND MOORE, M.J. Infectious salmon anaemia in New Brunswick: An historical perspective and update on control and management practices (1997-2002). Washington, DC U. S. Department of Agriculture Animal and Plant Health Inspection Service, U.S.Department of the Interior, U. S. Geological Survey, and U.S.Department of Commerce, National Marine Fisheries Service. In: Miller, Otis; Cipriano, Rocco C., tech.coords.International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA.Tech.Bull.2003; 1902: 145-153

177. MCGUIRE, T. C., ADAMS, D. S., JOHNSON, G. C., KLEVJER-ANDERSON, P., BARBEE, D. D., GORHAM, J. R.: Acute arthritis in caprine arthritis-encephalitis virus challenge exposure of vaccinated or persistently infected goats. Am. J. Vet. Res. 1986; 47: 537-540

178. MCLNERNEY, T. L., MCLAIN, L., ARMSTRONG, S. J., DIMMOCK, N. J: A human IgG1 (b12) specific for the CD4-binding site of HIV-1 neutralizes by inhibiting the virus fusion entry process, but b12 Fab neutralizes by inhibiting a post fusion event. Virology 1997; 233: 313-326

179. MERRIL, P.L. A comparative review of diagnostic assays used to detect infectious salmon anaemia virus in the United States. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp25-37

180. MI, J., LI, Z. Y., NI, S., STEINWAERDER, D., LIEBER, A.: Induced apoptosis supports spread of adenovirus vectors in tumors. Hum. Gene Ther. 2001; 12: 1343-1352

181. MIKALSEN AB, TEIG A, H. A., MJAALAND S, RIMSTAD E: Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon *Salmo salar* 47: 175-181. Dis Aquat Org 2001; 47: 175-181

182. MILLER O, CIPRIANO, R.C.: International response to infectious salmon anemia: prevention, control, and eradicationproceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp194

183. MJAALAND, S., RIMSTAD, E., CUNNINGHAM, C.O.: Molecular diagnosis of

infectious salmon anaemia. In: Cunningham CO (ed) Reviews: Methods and Technology in Fish Biology and Fisheries 2002.

184. MJAALAND, S., RIMSTAD, E., FALK, K., DANNEVIG, B. H.: Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. J Virol 1997; 71: 7681-7686

185. MONEKE, E.E, KIBENGE, M.J.T, GROMAN, D., JOHNSON, G.R., IKEDE, B.O., KIBENGE, F.S.B.: Demonstration of infectious salmon anaemia virus (ISAV) RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with ISAV. J Vet Diag Invest 2003; 15: 407-417

186. MONTEFIORI, D., MOORE, J. P.: HIV vaccines. Magic of the occult? Science 1999; 283: 336-337

187. MONTEFIORI, D. C., ROBINSON, W. E., Jr., HIRSCH, V. M., MODLISZEWSKI, A., MITCHELL, W. M., JOHNSON, P. R.: Antibody-dependent enhancement of simian immunodeficiency virus (SIV) infection in vitro by plasma from SIV-infected rhesus macaques. J. Virol. 1990; 64: 113-119

188. MORENS, D. M., VENKATESHAN, C. N., HALSTEAD, S. B.: Dengue 4 virus monoclonal antibodies identify epitopes that mediate immune infection enhancement of dengue 2 viruses. J. Gen. Virol. 1987; 68: 91-98

189. MORENS, D. M., HALSTEAD, S. B.: Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies. J. Gen. Virol. 1990; 71: 2909-2914

190. MORRIS, S. J., PRICE, G. E., BARNETT, J. M., HISCOX, S. A., SMITH, H., SWEET, C.: Role of neuraminidase in influenza virus-induced apoptosis. J. Gen. Virol. 1999; 80: 137-146

191. MULLINS, J .E., GROMAN, D., WADOWSKA, D.: Infectious salmon anemia in salt water Atlantic salmon in New Brunswick, Canada. Bull. Eur. Ass. Fish Pathol. 1998; 18: 110-115

192. MUNIR, K., KIBENGE, F. S. B.: Detection of infectious salmon anaemia virus by real-time RT-PCR. J Virol Methods.2004;117: 37-47

193. MURRAY, A.G.: The epidemiology of infectious salmon anaemia in Scotland. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; Tech. Bull. 1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection

Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; 55-62

194. MURRAY, A. G., SMITH, R. J., STAGG, R. M.: Shipping and the spread of infectious salmon anemia in Scottish aquaculture. Emerg Infect Dis 2002; 8 1-5

195. MUZIO, M., CHINNAIYAN, A.M., KISCHKEL, F.C., O'ROURKE, K., SHEVCHENKO, A., NI, J., SCAFFIDI, C., BRETZ, J. D., ZHANG, M., GENTZ, R., MANN, M., KRAMMER, P. H., PETER, M. E., DIXIT, V. M.: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell 1996; 85: 817-827

196. MYERS, R. M., MANIATIS, T., LERMAN, L. S.: Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol. 1987; 155: 501-527

197. NEMEROFF, M. E., BARABINO, S. M., LI, Y., KELLER, W., KRUG, R. M.: Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. Mol. Cell 1998; 1: 991-1000

198. NOBUSAWA, E., AOYAMA, T., KATO, H., SUZUKI, Y., TATENO, Y., NAKAJIMA, K.: Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 1991; 182: 475-485

199. NYLUND, A., HOVLAND, T., HODNELAND, F., NILSEN, L. P.: Mechanisms for transmission of infectious salmon anemia (ISA). Dis Aquat Org 1994; 19: 95-100

200. NYLUND A, KVENSETH AM, KROSSOY B: Susceptibility of wild salmon (*Salmo salar* L.) to infectious salmon anaemia (ISA). Bull Euro Ass Fish Pathol 1995a; 15: 152-156

201. NYLUND, A., HOVLAND, T., WATANABE, ENDRESEN, C.: Presence of infectious salmon anemia virus (ISAV) in tissue of Atlantic salmon, *Salmo salar* L., collected during three separate out breaks of the disease. J. Fish Dis. 1995b; 18:135-145

202. NYLUND, A., JACOBSE, P.: Sea trout (*Salmo trutta*): a carrier of infectious salmon anemia virus (ISAV). J. Fish Biol. 1995; 174-176

203. NYLUND, A., KROSSOY, K., WATANABE, K., HOLM, J.A.: Target cells for the ISA virus in Atlantic salmon (*Salmo salar* L.). Bulletin of the European Association of Fish Pathologists 1996; 16: 72

204. NYLUND, A.: Reservoir species for the infectious salmon anemia virus (ISAV).

Proceedings from workshop on Infectious salmon anemia, St Andrews, New Brunswick 29, 1997.

205. NYLUND, A., DEVOLD, M., MULLINS, J., PLARRE, H.: Herring (*Clupea harengus*): a host for infectious salmon anaemia virus (ISAV). Bull Euro Ass Fish Pathol 2002; 22: 311-318

206. OCHIAI, H., KUROKAWA, M., MATSUI, S., YAMAMOTO, T., KUROKI, Y., KISHIMOTO, C., SHIRAKI, K.: Infection enhancement of influenza A NWS virus in primary murine macrophages by anti-hemagglutinin monoclonal antibody. J. Med. Virol. 1992; 36: 217-221

207. OLSEN, Y.A., FALK, K., REITES.: Cortisol and lactate levels in Atlantic Salmon developing infectious salmon anemia (ISA). Dis. Aquat. Org. 1992; 14: 99-104

208. OLSEN, C., SCOTT, F.: Evaluation of antibody-dependent enhancement of feline infectious peritonitis virus infectivity using in situ hybridization. Microb. Pathog. 1993; 14: 275-285

209. OLSEN, C. W., CORAPI, W. V., NGICHABE, C. K., BAINES, J. D., SCOTT, F. W.: Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J. Virol. 1992; 66: 956-965

210. OLSEN, C.W., KEHREN, J.C., DYBDAHL-SISSOKO, N.R., HINSHAW, V.S. bcl-2 Alters influenza virus yield, spread, and haemagglutinin glycosylation. J Virol. 1996; 70: 663-666

211. OUTLAW, M. C., DIMMOCK, N. J.: Mechanisms of neutralization of influenza virus on mouse tracheal epithelial cells by mouse monoclonal polymeric IgA and polyclonal IgM directed against the viral haemagglutinin. J. Gen. Virol. 1990; 71: 69-76

212. OUTLAW, M. C., ARMSTRONG, S. J., DIMMOCK, N. J.: Mechanisms of neutralization of influenza virus in tracheal epithelial and BHK cells vary according to IgG concentration. Virology 1990; 178: 478-485

213. OUTLAW, M. C., DIMMOCK, N. J.: IgG neutralization of type A influenza viruses and the inhibition of the endosomal fusion stage of the infectious pathway in BHK cells. Virology 1993; 195: 413-421

214. OYE, A. K., RIMSTAD, E.: Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. Dis. Aquat. Organ 2001; 48: 1-5

215. PAABO, S., SEVERINSSON, L., ANDERSSON, M., MARTENS, I., NILSSON, T., PETERSON, P. A.: Adenovirus proteins and MHC expression. Adv. Cancer Res. 1989; 52: 151-163

216. PAHL, H. L.: Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol Rev. 1999; 79: 683-701

217. PARK, Y. W., WILUSZ, J., KATZE, M. G.: Regulation of eukaryotic protein synthesis: selective influenza viral mRNA translation is mediated by the cellular RNA-binding protein GRSF-1. Proc. Natl. Acad. Sci. U. S. A 1999; 96: 6694-6699

218. PAROLI, M., SCHIAFFELLA, E., DI ROSA, F., BARNABA, V.: Persisting viruses and autoimmunity. J. Neuroimmunol. 2000; 107: 201-204

219. PARREN, P. W., BURTON, D. R.: The antiviral activity of antibodies in vitro and in vivo. Adv. Immunol. 2001; 77: 195-262

220. PEIRIS, J. S., PORTERFIELD, J. S.: Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. Nature 1979; 282: 509-511

221. PEIRIS, J. S., PORTERFIELD, J. S.: Antibody-dependent enhancement of plaque formation on cell lines of macrophage origin - a sensitive assay for antiviral antibody. J. Gen. Virol. 1981; 57: 119-125

222. PERNO, C. F., BASELER, M. W., BRODER, S., YARCHOAN, R.: Infection of monocytes by human immunodeficiency virus type 1 blocked by inhibitors of CD4-gp120 binding, even in the presence of enhancing antibodies. J. Exp. Med. 1990; 171: 1043-1056

223. PORTELA, A., DIGARD, P.: The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J. Gen. Virol.2002; 83: 723-734

224. PORTER, A. G.: Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. J. Virol. 1993; 67: 6917-6921

225. R, D.D., LARSEN, A.D, PORTER, H.G. The pathogenesis of Aleutian disease of mink. Enhancement of tissue lesions following the administration of a killed virus vaccine or passive antibody. J. Immunol. 1972; 109:1-7

226. R, D.D., LARSEN, A.D, PORTER, H.G. Aleutian disease of mink. Adv immunol. 1980; 29: 261-286

227. POSSEE, R. D., SCHILD, G. C., DIMMOCK, N. J.: Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-

haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity. J. Gen. Virol. 1982; 58: 373-386

228. PRABHAKAR, B. S., NATHANSON, N.: Acute rabies death mediated by antibody. Nature 1981; 290: 590-591

229. PROHASZKA, Z., NEMES, J., HIDVEGI, T., TOTH, F. D., KEREKES, K., ERDEI, A., SZABO, J., UJHELYI, E., THIELENS, N., DIERICH, M. P., SPATH, P., GHEBREHIWET, B., HAMPL, H., KISS, J., ARLAUD, G., FUST, G.: Two parallel routes of the complement-mediated antibody-dependent enhancement of HIV-1 infection. AIDS 1997; 11: 949-958

230. QIAN, X. Y., ALONSO-CAPLEN, F., KRUG, R. M.: Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. J. Virol.1994; 68: 2433-2441

231. QIAN, X. Y., CHIEN, C. Y., LU, Y., MONTELIONE, G. T., KRUG, R. M.: An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. RNA. 1995; 1: 948-956

232. QIU, Y., NEMEROFF, M., KRUG, R. M.: The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. RNA. 1995; 1: 304-316

233. RAO, R. V., HERMEL, E., CASTRO-OBREGON, S., DEL RIO, G., ELLERBY, L. M., ELLERBY, H. M., BREDESEN, D. E.: Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J. Biol. Chem. 2001; 276: 33869-33874

234. RAYNARD, R. S., MURRAY, A. G., GREGORY, A.: Infectious salmon anemia virus in wild fish from Scotland. Dis Aquat Org 2001a; 46: 93-100

235. RAYNARD, R.S., SNOW, M., BRUNO, D.W.: Experimental infection models and susceptibility of Atlantic salmon *Salmo salar* to a Scottish isolate of infectious salmon anaemia virus. Dis Aquat Org 2001; 47: 169-174

236. RIMSTAD, E., FALK, K., MIKALSEN, A.B., TEIG, A.: Time course tissue distribution of infectious salmon anaemia virus in experimentally infected Atlantic salmon *Salmo salar*. Dis Aquat Org 1999; 36: 107-112

237. RIMSTAD, E., MJAALAND, S., SNOW, M., .MIKALSEN, A.B., CUNNINGHAM, C.O.: Characterization of the infectious Salmon Anemia Virus Genomic Segment That Encodes the Putaitve Haemagglutinin. J Virol 2001; 75: 5352-5356

238. RIMSTAD, E., MJAALAND, S.: Infectious salmon anaemia virus - An orthomyxovirus

causing an emerging infection in Atlantic salmon. Apmis 2002; 110: 273-282

239. RITCHIE, R.J., COOK, M., KRISTA MELVILLE.: Identification of infectious salmon anemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain difference. Dis Aquat Org 2001; 44: 171-178

240. RITCHIE, R. J., HEPPELL, J., COOK, M. B., JONES, S., GRIFFITHS, S. G.: Identification and characterization of segments 3 and 4 of the ISAV genome. Virus Genes 2001 22: 289-297

241. RITCHIE, R. J., BARDIOT, A., MELVILLE, K., GRIFFITHS, S., CUNNINGHAM, C. O., SNOW, M.: Identification and characterisation of the genomic segment 7 of the infectious salmon anaemia virus genome. Virus Res. 2002; 84: 161-170

242. ROBINSON, W. E., Jr., MONTEFIORI, D. C., MITCHELL, W. M.: Antibody-dependent enhancement of human immunodeficiency virus type 1 infection. Lancet 1988; 1: 790-794

243. ROBINSON, W. E., Jr., MONTEFIORI, D. C., MITCHELL, W. M.: Complement-mediated antibody-dependent enhancement of HIV-1 infection requires CD4 and complement receptors. Virology 1990; 175: 600-604

244. RODGER, H. D., RICHARDS, R. H.: Haemorrhagic smolt syndrome: a severe anaemic condition in farmed salmon in Scotland. Vet Rec 1998; 142: 538-541

245. ROITE, I. J., BROSTOFF, MALE, D. K.: Cell-mediated immunity. In: *Immnunology* Gower Medical Publishing Ltd, New York, 1985, pp. 1-2.

246. ROIVAINEN, M., PIIRAINEN, L., RYSA, T., NARVANEN, A., HOVI, T.: An immunodominant N-terminal region of VP1 protein of poliovirion that is buried in crystal structure can be exposed in solution. Virology 1993; 195: 762-765

247. ROLLAND, J.B., NYLUND, A.: Sea running brown trout: carrier and transmitter of the infectious salmon anemia virus (ISAV) 18: 50-55. Bull Euro Ass Fish Pathol 1999; 18: 50-55

248. ROLLAND, J.B, BOUCHARD, D.A, WINTON, J.R. Improved diagnosis of infectious salmon anaemia virus by use of a new cell line derived from Atlantic salmon kidney tissue. In: Miller, Otis; Cipriano, Rocco C., tech. coords. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp63-68

249. ROLLAND, J.B., WINTON, J.R.: Relative resistance of pacific salmon to

infectioussalmon anaemia virus. 28th Annual Eastern Fish Health Workshop 2004.

250. ROULSTON, A., MARCELLUS, R. C., BRANTON, P. E.: Viruses and apoptosis. Annu. Rev. Microbiol. 1999; 53: 577-628

251. SAKAHIRA, H., ENARI, M., NAGATA, S.: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 1998; 391: 96-99

252. SALONIUS, K., BARTON, T., ERDAL, J.I. Efficacy of a multivalent vaccine against cohabitation challenge with Norwegian ISAV. [3rd International Symposium on Fish Vaccinology, Bergen, Norway, April 9-11]. 2003.

253. SANCHEZ, L., ABUIN, M., AMARO, R.: Cytogenetic characterization of the AS cell line derived from the Atlantic salmon (*Salmo salar* L.). Cytogenet Cell Genet 1993; 64: 35-38

254. SANDVIK, T., RIMSTAD, E., MJAALAND, S.: The viral RNA 3'- and 5'-end structure and mRNA transcription of infectious salmon anaemia virus resemble those of influenza viruses. Arch. Virol. 2000; 145: 1659-1669

255. SCAFFIDI, P., MISTELI, T., BIANCHI, M. E.: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002; 418: 191-195

256. SCHLESINGER, J. J., BRANDRISS, M. W.: Antibody-mediated infection of macrophages and macrophage-like cell lines with 17D-yellow fever virus. J. Med. Virol. 1981; 8: 103-117

257. SCHULTZ, D. R., HARRINGTON, W. J., Jr.: Apoptosis: programmed cell death at a molecular level. Semin. Arthritis Rheum. 2003; 32: 345-369

258. SCHULTZ-CHERRY,S., DYBDAHL-SISSOKO, N., NEUMANN, G., KAWAOKA, Y., HINSHAW, V.S. Influenza virus NS1 protein induces apoptosis in cultured cells. J Virol.2001.2001; 75: 7875-7881

259. SCHUTTEN, M., ANDEWEG, A. C., BOSCH, M. L., OSTERHAUS, A. D.: Enhancement of infectivity of a non-syncytium inducing HIV-1 by sCD4 and by human antibodies that neutralize syncytium inducing HIV-1. Scand. J. Immunol. 1995; 41: 18-22

260. SCHUTTEN, M., ANDEWEG, A. C., RIMMELZWAAN, G. F., OSTERHAUS, A. D.: Modulation of primary human immunodeficiency virus type 1 envelope glycoprotein-mediated entry by human antibodies. J. Gen. Virol. 1997; 78: 999-1006

261. SHISLER, J., YANG, C., WALTER, B., WARE, C. F., GOODING, L. R.: The

adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. J. Virol. 1997; 71: 8299-8306

262. SHISLER, J. L., SENKEVICH, T. G., BERRY, M. J., MOSS, B.: Ultraviolet-induced cell death blocked by a selenoprotein from a human dermatotropic poxvirus. Science 1998; 279: 102-105

263. SKEHEL, J. J., WILEY, D. C.: Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. 2000; 69: 531-569

264. SMITH, T. J., OLSON, N. H., CHENG, R. H., LIU, H., CHASE, E. S., LEE, W. M., LEIPPE, D. M., MOSSER, A. G., RUECKERT, R. R., BAKER, T. S.: Structure of human rhinovirus complexed with Fab fragments from a neutralizing antibody. J. Virol. 1993; 67: 1148-1158

265. SNOW M, RAYNARD R, BRUNO DW, VAN NIEUWSTADT AP, OLESEN NJ, LOEVOLD T, WALLACE C: Investigation into the susceptibility of saithe Pollachius virens to infectious salmon anaemia virus (ISAV) and their potential role as a vector for viral transmission. Dis Aquat Org 2002; 50: 13-18

266. SNOW, M., CUNNINGHAM, C. O.: Characterisation of the putative nucleoprotein gene of infectious salmon anaemia virus (ISAV). Virus Res 2001a; 74: 111-118

267. SNOW, M., RAYNARD, R. S., BRUNO, D. W.: Comparative susceptibility of Arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. Aquaculture 2001; 196: 47-54

268. SNOW, M., RITCHIE, R., ARNAUD, O., VILLOING, S., ASPEHAUG, V., CUNNINGHAM, C. O.: Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. Virus Res. 2003; 92: 99-105

269. SOMMER, A. I., MENNEN, S.: Multiplication and haemadsorbing activity of infectious salmon anaemia virus in the established Atlantic salmon cell line. J Gen Virol 1997; 78: 1891-1895

270. SPEILBERG, L., EVENSEN, O., DANNEVIG, B. H.: A sequential study of the light and electron microscopic liver lesions of infectious anemia in Atlantic salmon (*Salmo salar* L.). Vet. Pathol. 1995; 32: 466-478

271. STARK, G. R., KERR, I. M., WILLIAMS, B. R., SILVERMAN, R. H., SCHREIBER, R. D.: How cells respond to interferons. Annu. Rev. Biochem. 1998; 67: 227-264

272. STUECKEMANN, J. A., HOLTH, M., SWART, W. J., KOWALCHYK, K., SMITH, M. S., WOLSTENHOLME, A. J., CAFRUNY, W. A., PLAGEMANN, P. G.: Replication of lactate dehydrogenase-elevating virus in macrophages. 2. Mechanism of persistent infection in mice and cell culture. J. Gen. Virol. 1982; 59: 263-272

273. SU, H. L., LIAO, C. L., LIN, Y. L.: Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J. Virol. 2002; 76: 4162-4171

274. SULLIVAN, N. J.: Antibody-mediated enhancement of viral disease. Curr. Top. Microbiol. Immunol. 2001; 260: 145-169

275. TAKADA, A., FELDMANN, H., KSIAZEK, T. G., KAWAOKA, Y.: Antibody-dependent enhancement of Ebola virus infection. J. Virol. 2003; 77: 7539-7544

276. TAKEDA, A., TUAZON, C. U., ENNIS, F. A.: Antibody-enhanced infection by HIV-1 via Fc receptor-mediated entry. Science 1988; 242: 580-583

277. TAMURA, M., WEBSTER, R. G., ENNIS, F. A.: Antibodies to HA and NA augment uptake of influenza A viruses into cells via Fc receptor entry. Virology 1991; 182: 211-219

278. TAMURA, M., WEBSTER, R. G., ENNIS, F. A.: Neutralization and infection-enhancement epitopes of influenza A virus hemagglutinin. J. Immunol. 1993; 151: 1731-1738

279. TAMURA, M., WEBSTER, R. G., ENNIS, F. A.: Subtype cross-reactive, infection-enhancing antibody responses to influenza A viruses. J. Virol. 1994; 68: 3499-3504

280. TAN, S. L., GALE, M. J., Jr., KATZE, M. G.: Double-stranded RNA-independent dimerization of interferon-induced protein kinase PKR and inhibition of dimerization by the cellular P58IPK inhibitor. Mol. Cell Biol. 1998; 18: 2431-2443

281. TAYLOR, H. P., DIMMOCK, N. J.: Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. J. Exp. Med. 1985; 161: 198-209

282. THALI, M., MOORE, J. P., FURMAN, C., CHARLES, M., HO, D. D., ROBINSON, J., SODROSKI, J.: Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol.1993; 67: 3978-3988

283. THOMAS, A. A., VRIJSEN, R., BOEYE, A.: Relationship between poliovirus neutralization and aggregation. J. Virol. 1986; 59: 479-485

284. THORUD K, DJUPVIK: Infectious salmon anemia in atlantic salmon (*Salmo salar* L). Bull. Eur. Ass. Fish Pathol 1988; 8: 109-111

285. THORUD, K. Infectious salmon anaemia. Ph.D. Thesis. Norvegian college of veterinary medicine, Oslo, Norway. 1991

286. TIRADO, S. M., YOON, K. J.: Antibody-dependent enhancement of virus infection and disease. Viral Immunol. 2003; 16: 69-86

287. UGOLINI, S., MONDOR, I., SATTENTAU, Q. J.: HIV-1 attachment: another look. Trends Microbiol. 1999; 7: 144-149

288. VENNEMA, H., DE GROOT, R. J., HARBOUR, D. A., DALDERUP, M., GRUFFYDD-JONES, T., HORZINEK, M. C., SPAAN, W. J.: Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. J. Virol. 1990; 64: 1407-1409

289. VILLINGER, F., MAYNE, A. E., BOSTIK, P., MORI, K., JENSEN, P. E., AHMED, R., ANSARI, A. A.: Evidence for antibody-mediated enhancement of simian immunodeficiency virus (SIV) Gag antigen processing and cross presentation in SIV-infected rhesus macaques. J. Virol. 2003; 77: 10-24

290. VRIJSEN, R., MOSSER, A., BOEYE, A.: Postabsorption neutralization of poliovirus. J. Virol. 1993; 67: 3126-3133

291. WALLACE, M. J., SMITH, D. W., BROOM, A. K., MACKENZIE, J. S., HALL, R. A., SHELLAM, G. R., MCMINN, P. C.: Antibody-dependent enhancement of Murray Valley encephalitis virus virulence in mice. J. Gen. Virol. 2003; 84: 1723-1728

292. WALLACH, D., VARFOLOMEEV, E. E., MALININ, N. L., GOLTSEV, Y. V., KOVALENKO, A. V., BOLDIN, M. P.: Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. 1999; 17: 331-367

293. WANG, S. Z., RUSHLOW, K. E., ISSEL, C. J., COOK, R. F., COOK, S. J., RAABE, M. L., CHONG, Y. H., COSTA, L., MONTELARO, R. C.: Enhancement of EIAV replication and disease by immunization with a baculovirus-expressed recombinant envelope surface glycoprotein. Virology 1994; 199: 247-251

294. WEISS, R. C., SCOTT, F. W.: Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. Comp Immunol. Microbiol. Infect. Dis. 1981; 4: 175-189

295. WEISS, R. C., SCOTT, F. W.: Pathogenesis of feline infetious peritonitis: pathologic

296. WEISS, R. C., SCOTT, F. W.: Pathogenesis of feline infectious peritonitis: nature and development of viremia. Am. J. Vet. Res. 1981; 42: 382-390

297. WERGELAND, H. I., JAKOBSEN, R. A.: A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). Dis Aquat Org 2001; 44: 183-190

298. WILSON, M.R., WARR, G.W.: Fish immunoglobulins and the genes that encode them 201-221. Ann Rev Fish Dis 1992; 201-221

299. WRIGHT, K. E., WILSON, G. A., NOVOSAD, D., DIMOCK, C., TAN, D., WEBER, J. M.: Typing and subtyping of influenza viruses in clinical samples by PCR. J. Clin. Microbiol. 1995; 33: 1180-1184

300. WUDUNN, D., SPEAR, P. G.: Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 1989; 63: 52-58

301. WURZER, W. J., PLANZ, O., EHRHARDT, C., GINER, M., SILBERZAHN, T., PLESCHKA, S., LUDWIG, S.: Caspase 3 activation is essential for efficient influenza virus propagation. EMBO J. 2003; 22: 2717-2728

302. XU, X., NASH, P., MCFADDEN, G.: Myxoma virus expresses a TNF receptor homolog with two distinct functions. Virus Genes 2000; 21: 97-109

303. YALAMANCHILI, P., HARRIS, K., WIMMER, E., DASGUPTA, A.: Inhibition of basal transcription by poliovirus: a virus- encoded protease (3Cpro) inhibits formation of TBP-TATA box complex in vitro. J. Virol. 1996; 70: 2922-2929

304. YANG, J., LIU, X., BHALLA, K., KIM, C. N., IBRADO, A. M., CAI, J., PENG, T. I., JONES, D. P., WANG, X.: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997; 275: 1129-1132

305. YEWDELL, J. W., GERHARD,W.: Antigenic characterization of viruses by monoclonal antibodies. Annu. Rev. Microbiol. 1981; 35: 185-206

306. YUAN, H., YOZA, B. K., LYLES, D. S.: Inhibition of host RNA polymerase II-dependent transcription by vesicular stomatitis virus results from inactivation of TFIID. Virology 1998; 251: 383-392

307. ZAMBON, M. C.: The pathogenesis of influenza in humans. Rev. Med. Virol. 2001; 11: 227-241

308. ZHIRNOV, O. P., KONAKOVA, T. E., GARTEN, W., KLENK, H.: Caspase-dependent N-terminal cleavage of influenza virus nucleocapsid protein in infected cells. J.

308. ZHIRNOV, O. P., KONAKOVA, T. E., GARTEN, W., KLENK, H.: Caspase-dependent N-terminal cleavage of influenza virus nucleocapsid protein in infected cells. J. Virol. 1999; 73: 10158-10163

309. ZHIRNOV, O. P., KONAKOVA, T. E., WOLFF, T., KLENK, H. D. NS1 protein of influenza A virus down-regulates apoptosis. J. Virol. 2002a; 76: 1617-1625

310. ZHIRNOV, O. P., KSENOFONTOV, A. L., KUZMINA, S. G., KLENK, H. D.: Interaction of influenza A virus M1 matrix protein with caspases. Biochemistry (Mosc.) 2002b; 67: 534-539

Chapter 2

General materials and methods

2. General materials and methods

2.1. Viruses and cells

The selected strains of ISAV were propagated in CHSE-214, SHK-1 and TO cell lines. The IPNV strain FVX73 (ATCC) was propagated on CHSE-214 and TO cell lines. CHSE-214 cells were grown at 16°C in HMEM [Eagle's minimum essential medium containing Hank's salts] (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 1 x antibiotic-antimycotic solution (1000 units of penicillin G sodium, 1 mg of streptomycin sulphate, and 2.5 μg of amphotericin B) and 10% foetal bovine serum (FBS). The SHK-1 cells were grown at room temperature (22° C) in Leiboviotz L-15 medium (Invitrogen Life Technologies, Burlington, ON, Canada) as previously described (Kibenge et al., 2000). The TO cells were propagated in HMEM (BioWhittaker Inc., Walkersville, Maryland, USA) supplemented with 292 μg ml^{-1} L-glutamine (Sigma, Oakville, ON, Canada), 1% Non Essential Amino Acids (NEAA) (Sigma, Oakville, ON, Canada), 100 μg ml^{-1} gentamicin (Sigma, Oakville, ON, Canada) and 10% FBS (Wegerland & Jakobsen, 2001). For maintenance medium for all cell lines, FBS was reduced to 5%. Virus titration was performed on cell monolayers in 48-well plates and the virus titer in each cell line was determined from end point CPE by using the procedure described by Reed and Muench (1938). The virus titer was expressed as median tissue culture infectious dose ($TCID_{50}$) per 100 μl.

2.2. Indirect Fluorescent Antibody Test (IFAT)

TO or SHK-1 cells were grown in slide flasks (Fisher, Ottawa, ON, Canada) (3 ml/flask).

One-day old cell monolayers were infected with ISAV isolate NBISA01. The slides with infected and uninfected cells harvested at appropriate time intervals and fixed with fresh 4% paraformaldehyde prepared in1 x PBS for 10 minutes at room temperature. Slides were washed twice with 1 x PBS and then the cells were permeabilized by adding 0.2% Triton X-100 in PBS for 5 minutes at room temperature. The slides were washed three times with 0.2% Triton X-100 in PBS and then incubated with the primary antibody for 1 hr at room temperature. Slides were again washed with 0.2% Triton X-100 in PBS and then incubated with FITC conjugate (1:32) for 20 minutes at room temperature. The slides were washed once and examined under a fluorescent microscope.

2.3. Polyclonal antibody preparations.

The preparation of rabbit polyclonal antisera to purified ISAV isolates RPC-980-049(1) and RPC-990-002(4) has been described (Kibenge et al., 2000). The Atlantic salmon sera consisted of a pooled field sample collected from farmed fish in New Brunswick, Canada (Kibenge et al., 2002) and pooled serum collected from Atlantic salmon experimentally infected with ISAV strain NBISA01. The rainbow trout serum was also collected from fish experimentally infected with ISAV strain NBISA01. For the production of fish anti-ISAV sera, Atlantic salmon or rainbow trout were inoculated intraperitoneally with 10^5 $TCID_{50}$/0.2 ml of ISAV strain NBISA01 per fish, and kept in a freshwater tank at 10° C for at least 84 days. Normal rabbit serum, and sera from uninfected control rainbow trout and Atlantic salmon served as the negative control sera. Polyclonal rabbit anti-IPNV serum to IPNV strain FVX-8 used for the virus neutralization of IPNV was kindly provided by Dr. Carmencita

Yason (Regional Diagnostic Virology Services, Atlantic Veterinary College). All serum samples were heat-inactivated at 56 ° C for 30 minutes prior to use in order to destroy complement activity. This treatment also served to inactivate any ISAV (Falk et al., 1997) that might have contaminated the fish sera from prior infection.

2.4. Virus neutralization (VN) assay

The VN tests using the β method were carried out on 24-hour old cell monolayers in 48-well cell culture plates as previously described (Kibenge et al., 2001), with only slight modifications. Briefly, 100 µl of serial 2-fold dilutions of serum followed by an equal volume of virus suspension containing 100 $TCID_{50}$ of the virus were added to the cell monolayers drained of growth medium. The cell monolayers with the virus-antibody complex were incubated at room temperature for 1 hour before addition of 500 µl of fresh maintenance medium to each well. After a further 10 days incubation (14 days in case of the CHSE-214 cell line) at 16° C, cultures were examined microscopically for CPE to determine the VN test results. Virus neutralization titres were expressed as highest dilution of serum to completely neutralize 100 $TCID_{50}$ of the virus.

2.5. Virus purification

Virus was purified on a Ficoll-400 (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada) step gradient and a sucrose cushion as previously described (Kibenge et al., 2000). For virus purification, infected cell culture harvests were clarified at 3000 x g for 30 minutes in a JA14 Beckman rotor. The cell pellet was saved and suspended in 1 x TNE (10mMTris-

HCl, 0.1MNaCl, 1mM EDTA pH 7.5) buffer. Virus was precipitated twice with ammonium sulphate overnight. The virus precipitate was collected at 3000 x g and the pellets were resuspended in 1 x TNE buffer. The resuspended pellets were dialyzed against several changes of 1 x TNE buffer overnight. Virus suspension was layered on a Ficoll-400 (Amersham Pharmecia Biotech) step gradient consisting of 10% and 25% (w/w) Ficoll TNE and centrifuged at 95,000 x g for 1 hour at 4° C in an SW40 rotor. The virus band was collected from the interface and resuspended in 1 x TNE. Virus was then purified on a 30% sucrose cushion by centrifuging at 145,000 x g for 1hour at 4° C in an SW40 rotor. The virus band was collected and dialyzed against 1 x PBS overnight at 4° C.

2.6. References

1. FALK, K., NAMORK, E., RIMSTAD, E., MJAALAND, S., DANNEVIG, B. H.: Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). J Virol 1997; 71: 9016-9023

2. HONG, J.R., LIN, T.L., HSU, YA-LI., WU JEN-LEITH: Apoptosis precedes necrosis of fish cell line with infectious pancreatic necrosis virus infection. Virology 1998; 250: 76-84

3. KIBENGE, F. S., LYAKU, J. R., RAINNIE, D., HAMMELL, K. L.: Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. J Gen Virol 2000; 81: 43-150

4. KIBENGE, F. S. B., KIBENGE, M.J.T, MCKENNA, P.K., STOTHARD, P., MARSHAL R., CUSACK R.R., MCGEACHY, S.: Antigenic variation of isolates of infectious salmon anaemia virus correlates with the genetic variation of the viral heamagglutinin gene. J Gen Virol. 2001; 82: 2869-2879

5. KIBENGE, M.J.T., OPAZO, B., ROJAS, A., KIBENGE, F.S.B: Serological evidence of infectious salmon anaemia virus (ISAV) infection in farmed fishes, using an indirect enzyme-linked immunosorbent assay. Dis Aquat Org 2002; 51: 1-11

6. NICHOLS, J. E., MOCK, D. J. & ROBERTS, N. J. Use of FITC-labeled influenza virus

and flow cytometry to assess binding and internalization of virus by monocytes-macrophages and lymphocytes. Arch Virol. 1992;130: 441-445

7. REED, L. J. AND MUENCH, H. A: simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27: 493-497

8. WERGELAND, H. I., JAKOBSEN, R. A.: A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). Dis Aquat Org 2001; 44: 183-190

Chapter 3

Mechanism of cell death during infectious salmon anaemia virus infection of fish cell lines*

* **Joseph, T.**, Cepica, A., Brown, L., Ikede, B.O., and Kibenge, F.S. B. 2004. The mechanism of cell death during infectious salmon anaemia virus (ISAV) infection is cell-type specific. *Journal of General Virology. In Press.*

3.1. Summary

The damage caused by the viruses during replication in the host cells is known as CPE. Two general pathways which are known to cause viral CPE are apoptosis and necrosis. In this study, permissive fish cell lines SHK-1, CHSE-214, and TO were used to determine if ISAV-induced CPE is due to apoptosis or necrosis. Characteristic apoptotic DNA fragmentation was observed only in ISAV-infected SHK-1 and CHSE-214 cells. The apoptosis in ISAV-infected SHK-1 cells was confirmed by a fragment end-labeling assay suggesting that CPE in these cells is associated with apoptosis. ISAV-infected TO cells did not undergo apoptosis but showed leakage of high mobility group 1 (HMGB1) protein from the nucleus which is characteristic of cells undergoing necrosis. ISAV-infected SHK-1 cells did not show leakage of HMGB1 protein. Infection with two different strains of ISAV showed that the induction of apoptosis correlated with the appearance of CPE in SHK-1 cells. The ISAV-induced apoptosis was inhibited by a pan-caspase inhibitor Z-VAD-fmk indicating it is caspase dependent. The ISAV putative PB2 protein and translation products of RNA segment 7 specifically bound caspase-8 *in-vitro* suggesting that these viral proteins may have a role in the ISAV-induced apoptosis. These findings demonstrate for the first time that the mechanism of cell death during ISAV infection is dependent on the cell type, which may have implications for ISAV pathogenesis and persistence.

3.2. Introduction

Infectious salmon anaemia virus is known to cause overt and fatal systemic infection in farmed Atlantic salmon and asymptomatic infection in feral fish, a situation analogous to avian influenza viruses in domestic poultry and feral birds (reviewed by Kibenge et al., 2004). ISAV may infect and replicate in sea trout, brown trout, rainbow trout, eels, herring, and Arctic char, resulting in asymptomatic, probably life-long, carriers of the virus (reviewed by Kibenge et al., 2004). The mechanisms of pathogenesis and persistence of ISAV have not been studied well at the molecular level. In Atlantic salmon, ISAV seems to target leucocytic cells (Falk et al., 1995) and endothelial cells (Falk & Dannevig 1995) or endothelial-associated cells (Falk et al., 2001) and macrophages (Moneke et al., 2003). Permissive fish cell lines for ISAV include SHK-1 cells (Dannevig et al., 1995), TO cells (Wergeland & Jakobsen 2001), and ASK-2 cells (Rolland et al., 2002) which are macrophage-like cell lines (Dannevig et al., 1997; Wegerland & Jakobsen 2001; Rolland et al., 2002) in which virus replicates with production of CPE. Although all three cell lines are derived from the Atlantic salmon pronephros, they are distinctly different in their growth characteristics (media requirements, split ratio, cell composition), and they display distinctly different viral-induced CPE (Rolland et al., 2002; Kibenge et al., 2001). Some strains of ISAV can also replicate and cause CPE in CHSE -214 cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001). Virus replication also occurs in AS cell line (Sanchez et al., 1993) and Rtgill-W1 cell line (Bols et al., 1994) but in these later two cases, the virus is non-cytopathic.

The two pathways which are known to cause CPE and eukaryotic cell death during virus

infection *in-vivo* are apoptosis and necrosis. Morphological characteristics of necrosis include swelling and rapid cell degradation, disruption and loss of plasma membrane integrity accompanied by extensive cytoplasmic vacuolation (Gourdie, 1985). Necrosis is also characterized by the release of high mobility group 1 (HMGB1) protein, a chromatin-binding factor, from necrotic cells and the release of HMGB1 protein triggers inflammatory reactions that result in extensive tissue damage (Scaffidi et al., 2002). In contrast, apoptosis is an energy-dependent, tightly regulated process of cell death that occurs in response to either receptor-mediated (Fas, TNRF-1, DR3, DR4, and DR5) or non-receptor mediated (UV irradiation, DNA damage, granzymes) signals (Budiharjo et al., 1999). Apoptosis progresses through a series of morphological and biochemical changes including cytoplasmic shrinkage, chromatin condensation and intranucleosomal cleavage, phosphatidylserine exposure, plasma membrane blebbing, and cell fragmentation into apoptotic bodies that are phagocytosed by macrophages or other surrounding cells without provoking an inflammatory response (White, 1996; Vaux & Strasser 1996; O'Brien 1998; Bowen-Pope & Schaub 2001; Hay & Kannourakis 2002; Watanabe et al., 2002). Most, if not all, of these changes are effected by members of a family of cysteine proteases called caspases. Caspases include initiator caspases such as caspase-8 and -9 which cleave and activate other caspases, and effector caspase such as caspase-3, -6, and -7 which cleave a variety of cellular substrates, thereby disassembling cellular structures or inactivating enzymes within the nucleus of the affected cell (Thornberry & Lazebnik 1998; Watanabe et al., 2002).

A critical step of viral pathogenesis is the ability of a virus to inhibit host antiviral responses

through the inhibition of host gene expression and/or by interfering with the programmed cell death or apoptosis. Apoptosis of virus-infected cells can be due to virus-induced inhibition of host gene expression or due to the antiviral response of the host. Influenza A virus is known to induce apoptosis both *in-vivo* and *in-vitro* at the latest stages of infection, involving NA, M1, NS1, and PB1-F2 proteins (Zhirnov et al., 2002a, 2002b; Lowy 2003). The NS1 protein of influenza virus may also have an interferon dependent anti-apoptotic potential (Zhirnov et al., 2002b). As the role of apoptosis in ISAV pathogenesis has not been studied, examination of ISAV-induced apoptosis may provide a clearer picture of the cellular mechanisms of viral persistence and pathogenesis in ISAV infection. This study investigated the mechanism of cell death during ISAV infection of fish cell lines.

3.3. Material and methods

3.3.1. Chromosomal DNA fragmentation assay for apoptosis

Each well of a six well tissue culture plate (Costar, Corning, NY, USA) was seeded with three milliliters of growth medium containing approximately 1.5×10^5 cells/ml. Twenty four hour-old cell monolayers of TO, SHK-1, and CHSE-214 cells were infected with 1:10 dilution of the stock virus, ISAV isolate NBISA01, at an m.o.i of 10 in maintenance medium as described in section 2.1. Infected and uninfected control cells were harvested at 6, 12, and 18 hours and 1, 2, 3, 4, 5, and 6 days for TO cells and SHK-1 cells; and 1, 3, 5, 7, 9, 11, and 13 days post infection for CHSE-214 cells. In a repeat experiment, SHK-1 and TO cells infected with a less virulent strain of ISAV, isolate U5575-1, were harvested at 12 hours, and at day 1, 2, 3, 4, 5, 6, and 7 post infection. The duration of infection studied in each cell line

and virus were based on the development of CPE, determined in previous studies (Kibenge et al., 2000, 2001). In order to isolate cellular DNA the cell monolayer was washed two times with 1 x PBS at the end of each incubation time. The cells were then lysed with 500 µl of lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% Sarkosyl, to which 0.5 mg/ml Proteinase K is added before use) (Hong et al., 1999). The cells from two wells were pooled for each sample and then digested at 50°C for three hours. The cell digest was extracted once with an equal volume of phenol-chloroform-isoamyl (25:24:1) and once with chloroform-isoamyl. The aqueous phase was then precipitated with a 1/10 volume of 3 M Na-Acetate (pH 5.2) and a two times volume of 100% ethanol at -80°C overnight. The DNA pellet was washed with 70% ethanol, vacuum dried for eight minutes and resuspended in 36 µl of dd water. Before loading on to the gel 4 µl of RNaseA (1:10 dilution) was added. Cellular DNA preparation was then electrophoresed in 1.8% agarose gels at 70 V for four hours. The gels were stained with ethidium bromide and photographed under UV transillumination.

3.3.2. Fragment end labeling assay for apoptosis

SHK-1 and TO cells were grown in slide flasks (Nunc, VWR). After 24 hours, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i of 10 (section 2.1.). Slides with TO cells and SHK-1 cells were processed for fragment end labeling assay for apoptosis at 24 hours and 48 hours post-infection respectively. Cells were washed with 1 x PBS once before fixation with 4% paraformaldehyde for 10 minutes at room temperature. Fragment end labeling of DNA and staining of apoptotic cells were carried out using the TdT-FragEL DNA fragmentation detection kit (Oncogene, San Diego, CA, USA) according to manufacturer's

instructions. Briefly, terminal deoxynucleotidyl transferase (TdT) enzyme was used to add biotin-labeled and unlabeled deoxynucleotides (dNTPs) to the exposed 3' -OH ends of the DNA fragments generated in response to apoptotic signals. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine was used to generate an insoluble coloured substrate at the site of DNA fragmentation. The slides were mounted before viewing with a light microscope.

3.3.3. Indirect fluorescent antibody test (IFAT) for necrosis

SHK-1 and TO cells were grown in slide flasks (Nunc, VWR). After 24 hours, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i of 10. Slides with TO cells and SHK-1 cells were processed for IFAT for necrosis at 24 hours and 48 hours post infection respectively. The slides were fixed with fresh 4% paraformaldehyde for 10 minutes at room temperature. Slides were washed twice with 1 x PBS and then the cells were permeabilized by adding 0.2% Triton X-100 in PBS for five minutes at room temperature. The slides were washed three times with 0.2% Triton X-100 in PBS and then incubated with 1:50 dilution of anti-HMGB1 polyclonal IgG antibody (Abcam, Inc, Cambridge, MA, USA) for one hour at room temperature. Slides were again washed for five minutes with three changes of 0.2% Triton X-100 in PBS and then incubated with 1:40 dilution of anti-rabbit IgG (whole molecule) FITC conjugate (Sigma, Oakville, ON, Canada) for 20 minutes at room temperature. The slides were washed as described above and examined under a fluorescent microscope. Intensity of fluorescent staining in the nucleus was assessed by spot densitometry using AlphaDigiDoc™ AD-1200 software in the AlphaEase Fc imaging system

(Alpha Innotech Corporation, San Leandro, CA, USA). On the digital images of cells, two dimensional areas were created around the nuclei and fluorescence intensity was obtained through the corresponding pixel intensity values which were expressed as integrated density value. Thus, intensity of fluorescent staining in the nucleus of 30 cells from three different fields (10 cells/field) was measured. The difference between the mean integrated density values from the ISAV-infected and uninfected control cells was assessed using unpaired, 2-sample t-test.

3.3.4. Assay for caspase-3 activity

A colorimetric assay (Calbiochem, USA) was used to detect caspase-3-like activity in ISAV-infected cells. SHK-1 and TO cells were grown in growth medium at a concentration of 1 x 10^6 cells/well in six-well tissue culture plates (Nunc, VWR). After 24 hours, the cell monolayer was infected with ISAV strain NBISA01 at an m.o.i of 10 (section 2.1.). Infected cells and uninfected control cells were harvested at 6, and 12 hours and 1, 2, 4, and 6 days post-infection. The cell monolayer was washed two times with 1xPBS. The cells were trypsinized and then trypsin was neutralized with 1ml of growth medium. Cells were harvested by centrifugation at 1000 x *g* for 10 minutes, counted, and 1 x 10^6 cells were lysed with 50 µl of lysis buffer (Caspase-3 assay kit, Calbiochem, USA). The cell lysate was centrifuged at 10,000 x *g* for 10 minutes and the supernatant was collected and immediately frozen at -80°C until used. ELISA for caspase-3 activity was performed according to the manufacturer's instructions. ELISA using purified recombinant caspase-3 (Calbiochem, USA) served as the positive control.

3.3.5. Treatment of ISAV-infected cells with caspase inhibitor

SHK-1 cells were grown in 3 ml of growth medium (1.5 x 10^5 cells/ml) in 6-well tissue culture plates. After 24 hours the confluent cell monolayers were treated with the cell permeable preparation of a pan-caspase inhibitor Z-VAD-fmk at a concentration of 20 μM for four hours. Control cells were treated with DMSO (used as diluent of Z-VAD-fmk). After replacing with fresh medium containing caspase inhibitor, cells were infected with ISAV strain NBISA01 at an moi of 10 (section 2.1.). Cells were harvested after 4 days of infection when there was more than 50% CPE. Cellular DNA was isolated from virus-infected cells in the presence and absence of caspase inhibitor, and from uninfected control cells treated with either caspase inhibitor or DMSO. Isolated DNA was analysed for apoptotic DNA fragmentation by agarose gel electrophoresis as previously described (section 3.3.1).

3.3.6. Interaction of ISAV proteins with cellular caspases

Polystyrene microtiter plates (Falcon Pro Bind Assay plates, VWR) were used to investigate the interaction of ISAV proteins with caspase-8 following the method described by Zhirnov et al. (2002a), with minor modifications. For the production of various proteins of ISAV, open reading frames (ORFs) were amplified by RT-PCR and the PCR products were cloned into pCR®II vector having dual promoters, T7 and Sp6, in opposite orientation (Invitrogen Life Technologies, Burlington, ON, Canada). Plasmids were sequenced to check for correct orientation and to verify each viral cDNA sequence. Putative ISAV proteins were then produced by *in-vitro* transcription-translation reaction using the recombinant plasmids for the ten ISAV proteins and TNT™ T7/SP6 Coupled Reticulocyte Lysate System (TNT-RLS,

Promega, CA, USA). Use of ^{35}S-methionine in the *in-vitro* transcription-translation reaction enabled the analysis of putative ISAV proteins by SDS-PAGE and autoradiography (Kibenge et al., 2004). For use in ELISA, non-radioactive complete amino acid mixture was added instead of amino acid minus methionine and ^{35}S-methionine. The reaction components consisted of 25 µl TNT rabbit reticulocyte lysate, 2 µl TNT reaction buffer, 1 µl TNT reaction T7 or SP6 RNA polymerase, 0.5 µl amino acid mixture minus methionine, 0.5 µl amino acid mixture minus leucine, 1 µl of ribonuclease inhibitor, 2 µl of pCR®II vector containing open reading frames of ten putative ISAV proteins, and 18 µl nuclease-free water to a total volume of 50 µl. The reaction mixture was incubated at 37° C for 90 minutes. A non-related protein, luciferase also produced by similar *in-vitro* transcription-translation reaction, was used as negative control for putative ISAV proteins in ELISA.

For ELISA, each well of the microtiter plate was coated overnight with 15 µl of the reaction product in 85 µl of ELISA coating buffer (0.2 M Bicarbonate buffer, pH 9.6) at 4° C. Three replicates were set up for each protein. The microtiter well surface was then saturated with 3% goat serum prepared in 1 x T-PBS (Dulbecco's phosphate buffered saline with 0.05% (v/v) Tween 20) for one hour at room temperature. The caspase-8 (BioVision, California, USA) solution at a concentration of 2 units/100 µl in 1 x T-PBS containing 1% goat serum was then added to each well on the plate (100 µl/well) and incubated at 15° C for two hours. Rabbit polyclonal anti caspase-8 antibody (BioVision, California, USA) at a concentration of 1 µg/ml in 1 x T-PBS containing 1% goat serum was then added (100 µl/well) and the microtiter plate was further incubated. The concentrations of caspase-8 and anti-caspase-8

antibody for use were determined in a preliminary experiment by checker-board titrations of recombinant human caspase-8. The immune complex formed was detected by a reaction with secondary antibody conjugated to horseradish peroxidase (1:3000) (BioRad). Each step was separated by extensive washing using 1 x T-PBS. The substrate for horseradish peroxidase, ABTS (2,2'-azino-di-[3-ethylbezthiazoline-6-sulfonic acid]) (BioRad), was then added and the extent of the reaction was assessed by a colour intensity registered by an automated microtiter ELISA reader (SpectraMax™340) at 415 nm.

3.4. Results

3.4.1. Chromosomal DNA fragmentation assay for apoptosis

To determine whether cellular DNA of ISAV-infected cells undergoes fragmentation characteristic of apoptosis, DNA was isolated from SHK-1, CHSE-214, and TO cells at selected time-points after infection with ISAV and analyzed by gel electrophoresis. DNA fragmentation was observed in SHK-1 cells infected with ISAV strain NBISA01 beginning on day three post infection, and with ISAV strain U5575-1 beginning on day six post infection when CPE was apparent. The intensity of the DNA fragmentation increased (Fig.1a) as the CPE increased. No DNA fragmentation was observed in uninfected SHK-1 cells (Fig. 1b). DNA fragmentation was also observed in CHSE-214 cells infected with NBISA01 beginning on day nine post infection which corresponded with the appearance of CPE. No DNA fragmentation was observed in uninfected CHSE-214 cells. CHSE-214 cells infected with U5575-1 did not show CPE or DNA fragmentation. No DNA fragmentation was observed in ISAV-infected and uninfected TO cells although the infected cells showed

Figure 3. 1. CPE induced by ISAV strain NBISA01 in various fish cell lines. Panels **(a), (c),** and **(e)** are 24 hour old uninfected TO, SHK-1, and CHSE-214 cell monolayers, respectively. Panels **(b), (d),** and **(f)** are TO, SHK-1, and CHSE-214 cells showing CPE caused by ISAV strain NBISA01 at 4, 6, and 12 days post infection, respectively.

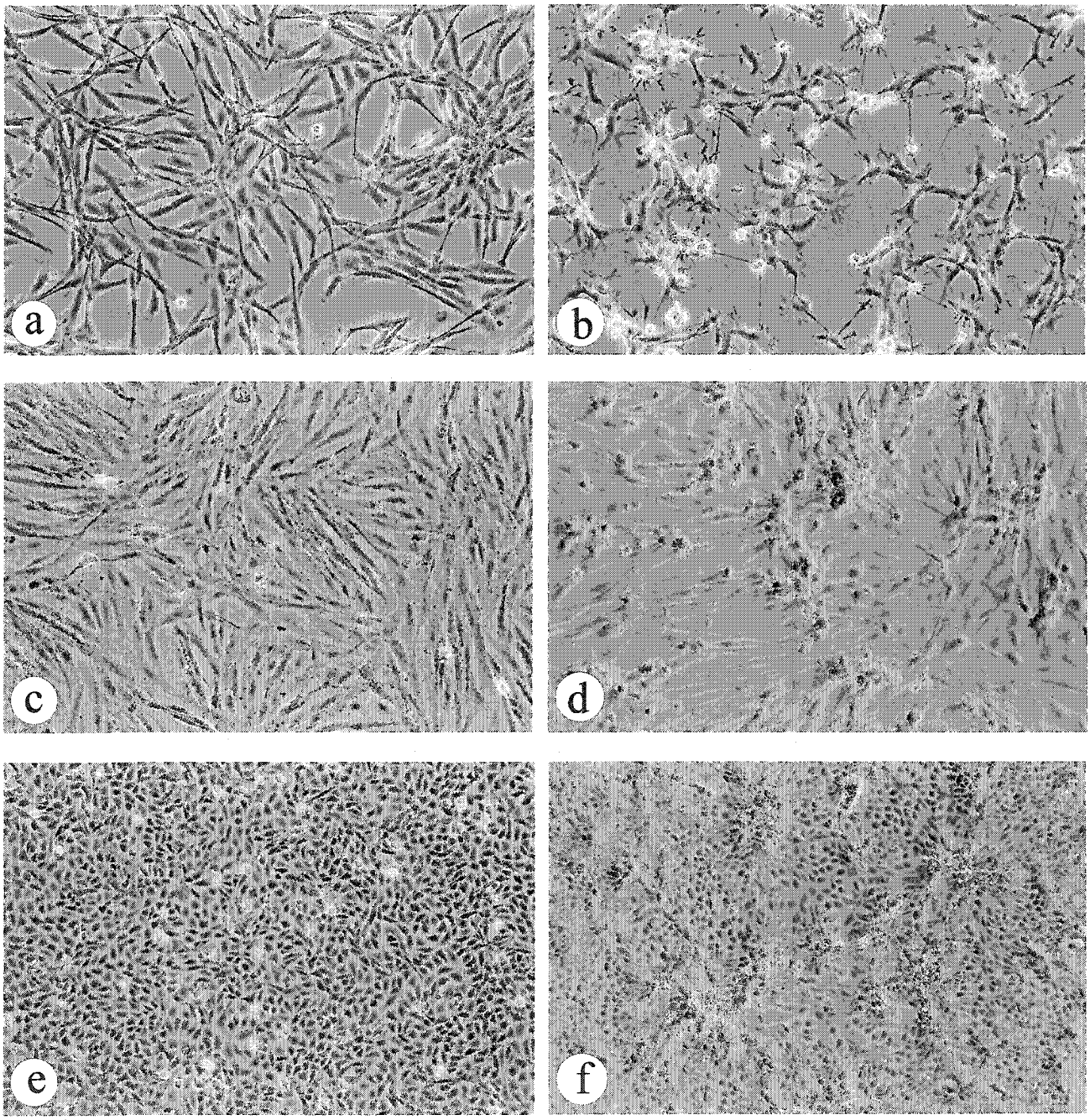


Fig. 3.1

Figure 3. 2. Agarose gel electrophoresis showing apoptosis in ISAV-infected cells. **(a)** SHK-1 cells infected with ISAV strain NBISA01 showing apoptotic DNA fragmentation. Lane 1: 1kb+ DNA ladder, Lanes 2, 3, and 4: DNA isolated from ISAV-infected SHK-1 cells at 3, 4, and 6 days post infection. **(b)** Uninfected SHK-1 cells. Lane 1:1kb+ DNA ladder, Lanes 2, 3, and 4: DNA isolated from uninfected control SHK-1 cells harvested at 3, 4, and 6 days.

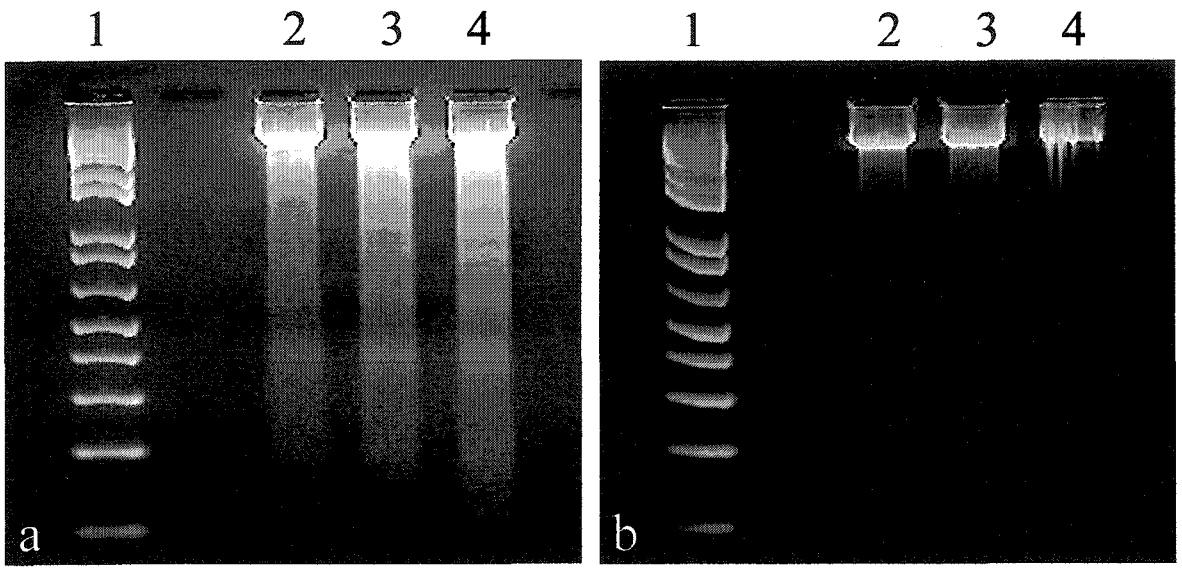


Fig. 3.2

CPE by day two with NBISA01 and by day four with U5575-1 which progressed to completion by 5-7 days post infection.

3.4.2. Fragment end labeling assay for apoptosis

Consistent with the above results, fragment end labeling assay also revealed apoptotic staining in the nucleus of ISAV infected SHK-1 cells (Fig. 3.3 b1, b2, b3). Extensive condensation of nuclei (Fig. 3.3 b1, b2, b3, arrows) was observed in the apoptotic cells when compared to that of intact rounded nuclei of uninfected cells (Fig. 3.3a). Using this assay apoptotic cells were detected in virus infected SHK-1 cells after 48 hours post-infection. No apoptotic staining was detected in either uninfected (Fig. 3.3c) or infected (Fig. 3.3d) TO cells.

3.4.3 Indirect fluorescent antibody test (IFAT) for necrosis

Previous studies have shown that HMGB1 protein is a specific marker for necrotic cells, as this protein leaks out rapidly into the extracellular space when membrane integrity is lost during necrosis (Scaffidi et al., 2002). Indirect fluorescent antibody test for HMGB1 protein showed bright fluorescence only in the nuclei of uninfected TO cells (Fig. 3.4a) whereas there was reduced fluorescence in the nuclei of ISAV-infected TO cells (Fig. 3.4b) indicative of HMGB1 protein leakage. Both uninfected (Fig. 3.4c) and ISAV-infected SHK-1 cells (Fig. 3d) did not show leakage of HMGB1 protein. Spot densitometry analysis showed that the reduction in fluorescence between ISAV-infected and uninfected TO cells was statistically significant (p-value = 0.018) (Fig. 3.5).

Figure 3. 3. Fragment end labeling of chromosomal DNA in cells undergoing apoptosis. Panel **(a)** is uninfected SHK-1 cells (160 x) after TdT-FragEL staining. Panels **(b1), (b2),** and **(b3)** are ISAV strain NBISA01 infected SHK-1 cells (250 x) undergoing apoptosis at 48 hours p.i. showing TdT-FragEL staining in the nucleus (arrows). Panel **(c)** is uninfected TO cells (160 x). Panel **(d)** is ISAV strain NBISA01 infected TO cells (160 x) (24 hours p.i) after TdT-FragEL staining.

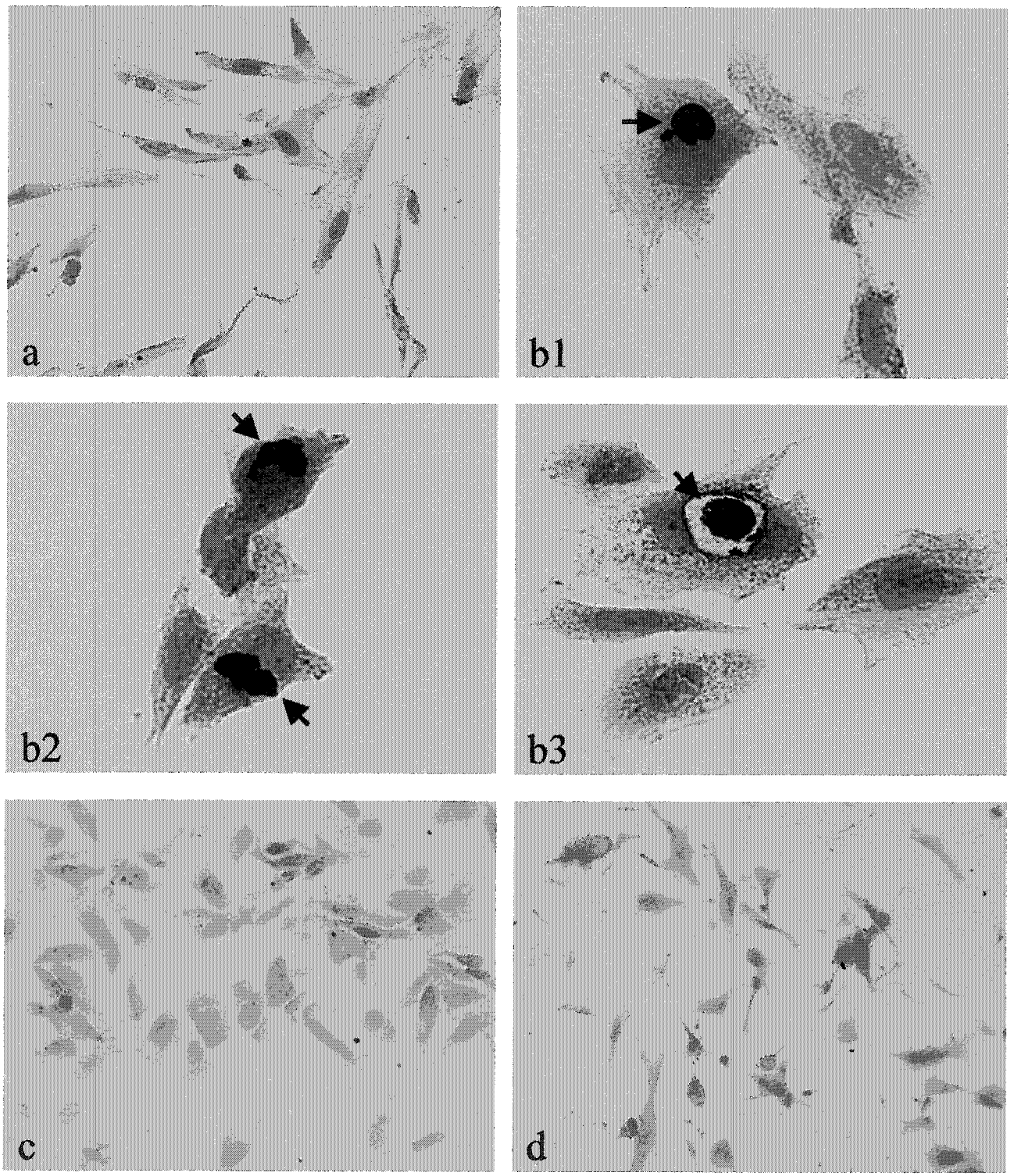


Fig. 3.3

Figure 3. 4. Release of HMGB1 protein during ISAV infection. Release of HMGB1 protein during ISAV infection. **(a)** Uninfected TO cells (250 x) showing nuclear localization of HMGB1 protein. **(b)** ISAV-infected TO cells (250 x) showing reduced fluorescent staining in the nucleus at 24 hours post infection. **(c)** Uninfected SHK-1 cells (250 x) showing nuclear localization of HMGB1 protein. **(d)** ISAV-infected SHK-1 cells (250 x) (48 hours post infection) undergoing apoptosis and showing nuclear localization of the HMGB1 protein.

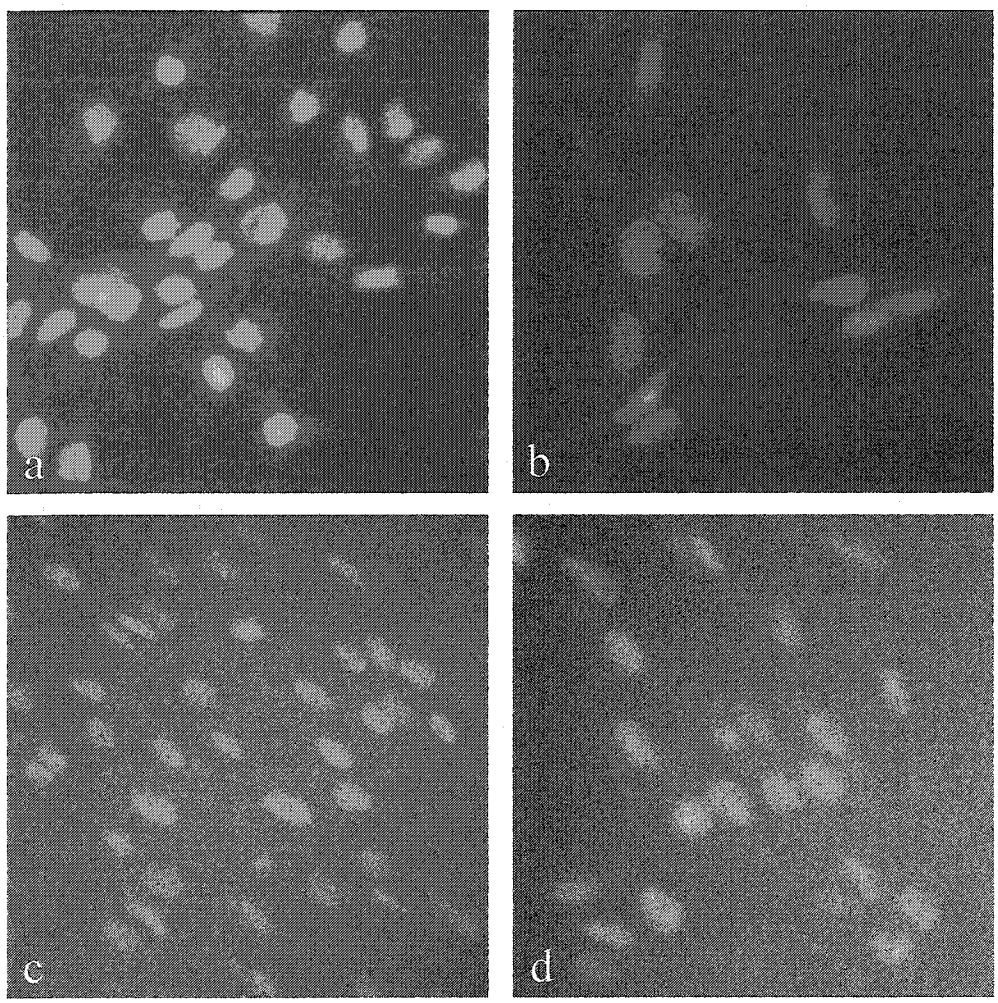


Fig. 3.4

Figure 3. 5. Quantitation of nuclear fluorescence of TO and SHK-1 cells following IFAT for HMGB-1 protein using spot densitometry. Fluorescence intensity was obtained through the corresponding pixel intensity values and expressed as integrated density value. Significant reduction in the fluorescence intensity was observed in the nucleus of ISAV-infected TO cells (p-value = 0.018) as compared to uninfected TO cells. No significant difference (p-value = 0.597) was observed between the fluorescence intensity of ISAV-infected SHK-1 cells undergoing apoptosis and uninfected SHK-1 cells.

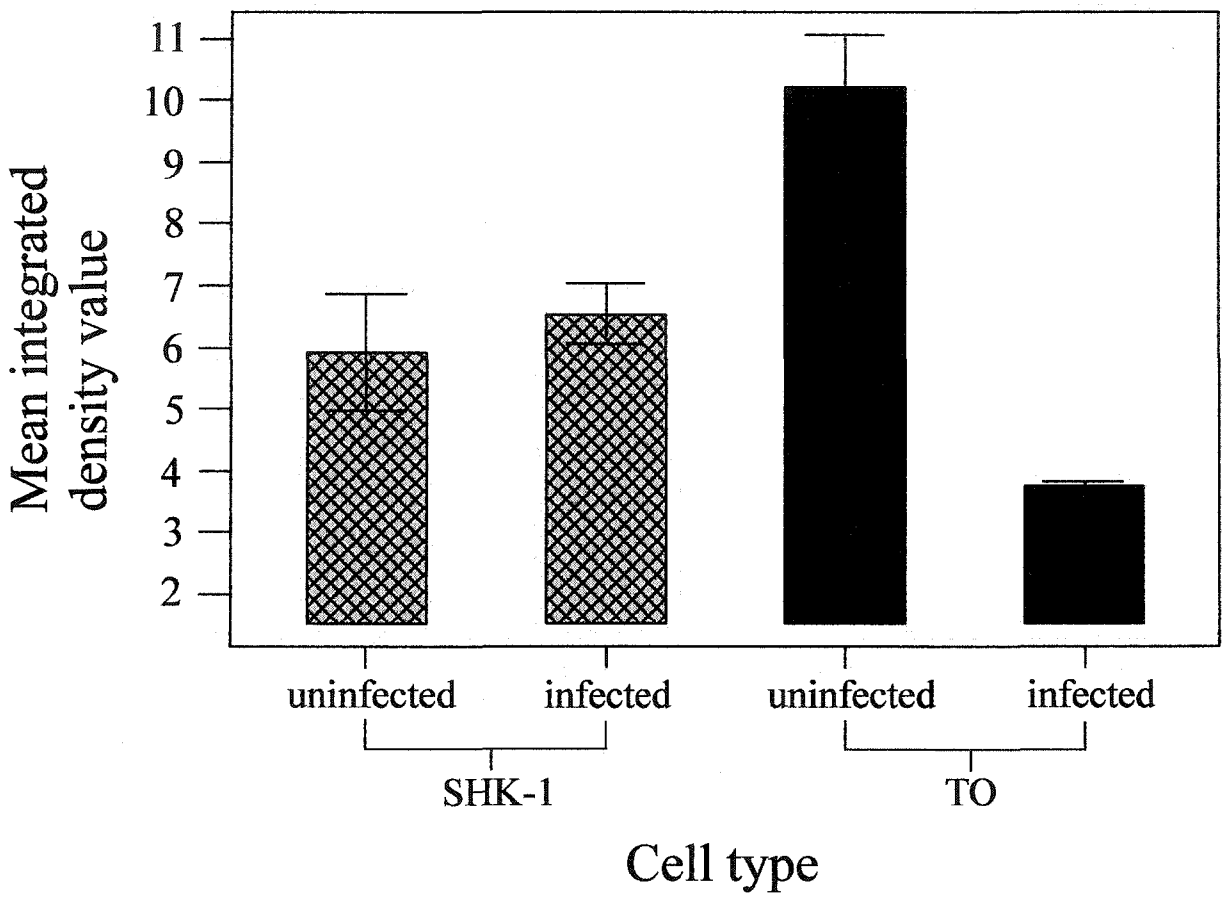


Fig. 3.5

There was no significant difference in the fluorescence observed between ISAV-infected and uninfected SHK-1 cells (p-value = 0.597) (Fig. 3.5).

3.4.4. Assay for caspase-3 activity

Caspase-3 is a central player in apoptosis regulation and its activity is often measured to determine the impact of a given apoptotic stimulus (Watanabe et al., 2002). Therefore a colorimetric assay was performed in order to determine caspase-3-like proteolytic activity in ISAV-infected SHK-1 and TO cells using a commercial kit (Calbiochem). No caspase-3-like activity was detected in either infected or control SHK-1 and TO cells.

3.4.5. Treatment of ISAV-infected cells with caspase inhibitor

In order to rule out involvement of other caspases in ISAV-induced apoptosis, a cell-permeable preparation of a pan-caspase inhibitor, Z-VAD-fmk, was used to determine if it could inhibit the induction of apoptosis in ISAV-infected SHK-1 cells. Use of pan-caspase inhibitor Z-VAD-fmk at a concentration of 20 μM inhibited apoptosis as well as CPE in ISAV-infected SHK-1 cells.

3.4.6. Interaction of ISAV proteins with cellular caspases

In order to establish if any of the ISAV proteins could interact with the caspase activation pathway, the eight RNA genomic segments of ISAV were expressed *in-vitro* with rabbit reticulocyte lysates and the products tested for binding with caspase-8, an upstream initiator caspase. Only three of the ten putative proteins of ISAV tested bound caspase-8. A specific,

strong positive signal was observed with the RNA segment 7 ORF2 product, and a weaker positive signal was observed with both RNA segment 7 ORF1 product and ISAV putative PB2 protein (Table 3.1).

3.5. Discussion

Viruses are capable of killing cells by necrosis or apoptosis during infection. The significance of virus-induced apoptosis is however not fully understood. In some cases the apoptotic cell death concomitantly brings on an abortion of the progeny virus production, suggesting that virus-induced apoptosis can have some role in the host defense mechanism against virus infection (Clem & Miller, 1994). Viruses can activate the apoptotic pathway by the direct action of specific viral proteins (eg. adenovirus E1A protein) (Roulston et al., 1999). Effects on cellular functions such as shutting down of protein synthesis or activation of pro-apoptotic proteins (e.g., up regulation of Fas in influenza virus infection) can also lead to apoptosis during virus infection (Balachandran et al., 2000). In some disease states, apoptosis occurs only in those cells that are infected with the virus (Lewis et al., 1996), whereas in other cases a virus-infected cell could cause apoptosis in an adjacent uninfected cell by a number of mechanisms including secretion of factors that activate the death program (Hardwick & Griffin 1997). A recent study by Chu & Ng (2003) documented that West Nile virus, can kill Vero cells by either apoptosis or necrosis, depending on the initial infectious dose.

In this study, the mechanism of cell death during ISAV infection was investigated using DNA fragmentation assay, fragment end labelling assay and a colorimetric assay for caspase 3-like

Table 3.1. Comparison of binding of caspase-8 by ISAV proteins

Protein used to coat ELISA microtiter plate	Optical density readings[a]	Interpretation
Active recombinant human caspase-8	0.709	Positive control
ISAV gene segment (ORF)		
1	0.0327 ±0.05	weak
7 (ORF 1)	0.0607 ±0.05	weak
7 (ORF 2)	0.1010 ±0.09	strong

[a]Mean OD readings after subtracting the mean OD of the negative control ± the standard error to the mean. 3% goat serum and luciferase were used as negative controls for caspase-8 and ISAV proteins which were expressed from the 10 ORFs in the 8 RNA segments of ISAV, respectively.

activity. In order to determine if CPE caused by ISAV is associated with apoptosis, three different permissive fish cell lines TO, SHK-1, and CHSE-214, were infected and analyzed for the fragmentation of cellular DNA that results in a characteristic "DNA ladder" when cells undergo apoptosis. During apoptosis, endonucleases are activated which cleave the DNA into fragments at sites between the nucleosomes, resulting in a ladder of evenly spaced DNA fragments of approximately 200 bp on an agarose gel (Collins et al., 1997). In necrosis, the DNA fragments are randomly cleaved and dispersed rather than concentrated in membrane-bound packages (Watanabe et al., 2002). The DNA fragmentation assay showed that ISAV strain NBISA01 induced apoptosis in SHK-1 cells at three days post-infection (Fig. 3. 2a) whereas a less virulent ISAV strain, isolate U5575-1, induced apoptosis by six days post-infection. ISAV strain NBISA01 which replicates in CHSE-214 cells with production of CPE (Fig. 3.1) (Kibenge et al., 2000) also induced apoptosis in infected CHSE-214 cells beginning at nine days post-infection.

Apoptotic DNA fragmentation in SHK-1 cells was also confirmed using fragment end labeling assay (Fig. 3. 3). In this technique terminal transferase enzyme is used to add labeled nucleotides (labeled dNTPs are used in fragment end labeling assay and labeled dUTP is used in TUNEL assay) to the 3' end of DNA fragments. The nucleotides were labeled with probes to permit detection by colorimetric assay (Gavieli et al., 1992). Using fragment end labeling assay, apoptotic cells were detected in ISAV infected SHK-1 cells at 48 hours post-infection (Fig. 3. 2a). In both SHK-1 and CHSE-214 cell lines, the intensity of DNA fragmentation increased as CPE progressed. This indicates that the CPE in these cells was due to virus-

induced apoptosis.

The absence of DNA fragmentation in ISAV-infected TO cells which developed complete CPE indicated that ISAV may kill TO cells by necrosis. In order to confirm this, the release of HMGB1 protein was studied. HMGB 1 protein is passively released only by necrotic or damaged cells when the membrane integrity is lost and the release of this protein triggers inflammatory reactions following necrosis (Scaffidi et al., 2002). Release of HMGB 1 protein from the nucleus is used a specific marker for necrosis since cells undergoing apoptosis do not release HMGB1 (Chu and Ng 2003; Scaffidi et al., 2002). Spot densitometry analyses showed a statistically significant reduction in the fluorescent staining of HMGB-1 protein in the nucleus of ISAV-infected TO cells (p-value = 0.018) (Fig. 3.4b, Fig. 3.5). These observations suggest that necrosis was the predominant mechanism of cell death in ISAV-infected TO cells in contrast to ISAV-infected SHK-1 cells which died by apoptosis. The uninfected TO and SHK-1 cells as well as SHK-1 cells undergoing apoptosis during ISAV infection retained this protein in their nuclei (Fig. 3.4 a, c, and d). Brighter fluorescence known to occur in the nucleus of cells undergoing apoptosis (Scaffidi et al., 2002), might have caused the slight but not statistically significant increase of fluorescence (p-value = 0.597; Fig. 3.5) in ISAV-infected SHK-1 cells. This observation further confirmed to us that ISAV induces apoptosis in SHK-1 cells and necrosis in TO cells.

Further investigation of the ISAV-induced apoptosis revealed that it was not associated with caspase-3-like activity. Caspase-3 is a cysteine protease activated in the terminal apoptosis

cascade. It is sequestered in the cytoplasm as a zymogen in its inactive form and activated when cleaved by other caspases. The caspase-3-like activity is often used as a quantitative measure of apoptosis. Since caspase-3 is part of the downstream effector caspases (Thornberry & Lazebnik 1998), its absence in ISAV-infected SHK-1 cells undergoing apoptosis could suggest that the apoptosis occurs independent of the caspase activation pathway. However, apoptosis in ISAV-infected SHK-1 cells could be blocked by a broad spectrum caspase inhibitor, Z-VAD-fmk. The tripeptide Z-VAD-fmk is an effective pan-caspase inhibitor (Leist & Jaattela 2001) which optimally inhibits caspase activity by fitting into the catalytic pocket of all caspases and inhibits them by cross linking the fluoromethylketone (fmk) group to the cysteine in the active site (Nicholson 1999). Alternatively, failure to detect caspase-3 could be because the caspase-3 assay reagents used were developed for use in mammalian cells and not fish cells. However, Guo et al. (2003) used a similar assay for caspase-3 like activity in sea bass (SB) cell lines and showed that activation of fish caspase-3 can be detected using assays developed for mammalian cells. Z-VAD-fmk has also been successfully used to inhibit virus-induced apoptosis in other fish cell lines such as Grunt Fin (GF) cells (Imajoh et al., 2004). Therefore, these findings suggest that ISAV-induced apoptosis in SHK-1 cells occurs via the caspase activation pathway but may not involve activation of caspase-3. Further studies showed that ISAV proteins, particularly the protein encoded by segment 7 ORF2, have the potential to specifically bind caspase-8 which might have implications in ISAV-induced apoptosis.

The absence of DNA fragmentation and release of HMGB1 protein in ISAV-infected TO

cells, which developed complete CPE, indicates that ISAV kills TO cells by necrosis and that the apoptosis induced by ISAV is cell-type specific. The TO cell line consists of uniformly epithelial-like cells (Wergeland & Jakobsen 2001), whereas CHSE-214 cell line consists of fibroblast-like cells and the SHK-1 cell line contains at least two subpopulations of cells one of which is fibroblast-like (Rolland et al., 2002). Only the ISAV-infected SHK-1 and CHSE-214 cells developed apoptosis. Although it is possible that the CPE observed *in-vitro* may not be relevant to events *in-vivo*, it is generally accepted that the execution of either apoptosis or necrosis in virus-infected cell cultures reflects the *in-vivo* pathogenicity of viruses (Hay & Kannourakis, 2002). Therefore it can be speculated that ISAV infection leads to total destruction of highly susceptible cells such as TO cells possibly by necrosis. Infection of such cell types *in-vivo* (for example leucocytes) may lead to inflammatory reactions due to the release of HMGB 1 protein and subsequent immune response and this may explain the clinical disease and pathology during a natural infection. At the same time, ISAV is capable of inducing apoptosis in cells such as SHK-1 and CHSE-214 cells. Infection of such cell types *in-vivo* may cause no inflammatory reactions, resulting in subclinical disease and virus persistence during a natural infection.

This study also suggested that ISAV proteins, particularly the protein encoded by segment 7 ORF2, have the potential to specifically bind caspase-8 (Table 3.1). Further investigation however is needed to conclusively prove the interaction of ISAV proteins with cellular caspases. Identification of the cellular factors involved in ISAV-induced apoptosis might lead to a better understanding of the mechanisms of persistence and pathogenesis of ISAV

infection *in-vivo*.

In conclusion, this study has demonstrated for the first time that ISAV is capable of inducing apoptosis, and the apoptosis is cell-type specific since it occurred only in ISAV-infected SHK-1 and CHSE-214. Further studies showed that ISAV induced a necrotic type of cell death in TO cells. These findings suggest that the mechanism of cell death during ISAV infection is dependent on the cell type, which may be important for the pathogenesis and persistence of ISAV in Atlantic salmon.

3.6. References

1. BALACHANDRAN, S., ROBERTS, P. C., KIPPERMAN, T., BHALLA, K.N., COMPANS, R.W., ARCHER, D.R., BARBER, G.N. α/β interferons potentiate virus-induced apoptosis through activation of the FADD/caspase-8 death signaling pathway. J. Virol. 2000; 74: 1513-1523

2. BOLS, N. C., GANASSIN, R.C., TOM, D. J. & LEE, L. E. Growth of fish cell lines in glutamine-free media. Cytotechnology 1994; 16: 159-66.

3. BOWEN-POPE, D. F. & SCHAUB, P. J. Apoptosis of smooth muscle cells is not silent: Fas/FADD initiates a program of inflammatory gene expression. Trends Cardiovasc Med 2001; 11: 42-45

4. BOUCHARD, D., KELEHER. W., OPITZ, H. M., BLAKE, S., EDWARDS, K. C. & NICHOLSON, B. L. Isolation of infectious salmon anaemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Dis Aquatic Org 1999; 35:131-137

5. BUDIHARDJO, I., OLIVER, H., LUTTER, M., LUO, X. & WANG, X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999; 15: 269–290

6. CHU, J. J. H. & NG, M. L. The mechanism of cell death during West Nile virus infection is dependent on initial infectious dose. J Gen Virol 2003; 84: 3305-3314

7. CLEM, R. J. & MILLER, L. K. Induction and Inhibition of apoptosis by insect viruses. In: apoptosis: The molecular basis of apoptosis in disease. Tomei, L. D. & Cope, F. O. (eds). Cold Spring Harbour Laboratory Press, New York, 1994; pp89-110

8. COLLINS, J.A., SCHANDI, C.A., YOUNG, K.K., VESELY, J., WILLINGHAM, M.C. Major DNA fragmentation is a late event in apoptosis. J. Histochem Cytochem. 1997; 45: 923-934

9. DANNEVIG, B. H., BRUDESETH, B. E., GJREN, T., RODE, M., WERGELAND, H. I., EVENSEN, Ø., & PRESS, C. M. Characterization of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 1997; 7: 213-226

10. DANNEVIG, B. H., FALK, K. AND NAMORK, E. Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol 1995; 76:1353-1359

11. FALK, K., JOHANSEN, M. B., FERGUSON, H. W., ASPEHAUG, V., KROSSøY, B., BIERING E. AND POPPE T. Immunohistochemical detection of infectious salmon anaemia virus (ISAV). Proceedings of European Association of Fish Pathologists, Dublin, September 2001; 10-14

12. FALK, K. AND DANNEVIG, B. H. Demonstration of infectious salmon anaemia (ISA) viral antigens in cell cultures and tissue sections. Virus Res 1995; 26: 499-504

13. FALK, K., PRESS, C. M., LANDSVERK, T. & DANNEVIG, B. H. Spleen and kidney of Atlantic salmon (*Salmo salar* L.) show histochemical changes early in the course of experimentally induced infectious salmon anaemia (ISA). Vet Immunol Immunopathol 1995; 49: 115-126

14. GAVRIELI, Y., SHERMAN, Y., BEN-SASSON, S. A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol. 1992; 119: 493-501

15. GOURDIE, R. B. Molecular and cellular pathology of tissue damage. In: Muir's Textbook of Pathology, 12th edn. Edited by J. R. Anderson. Baltimore, MD: Edward Arnold. 1985

16. GRIFFITHS, S., COOK, M., MALLORY, B. AND RITCHIE, R. Characterization of ISAV proteins from cell culture. Dis Aquat Org 2001; 45: 19-24

17. GUO, Y. X., WEI, T., DALLMANN K, AND KWANG, J. Induction of caspase-dependent apoptosis by betanodaviruses GGNNV and demonstration of protein α as an apoptosis inducer. Virology 2003; 308: 74-82

18. HARDWICK, J. M. AND GRIFFIN, D. E. Viral effects on cellular functions. In: Viral pathogenesis, Lippincott-Raven Publishers, Philadelphia, USA. 1997, pp 55-79

19. HAY, S. AND KANNOURAKIS, G. A time to kill: viral manipulation of the cell death program. J Gen Virol 2002; 83: 1547-1564

20. IMAJOH, M., SUGIURA, H., OSHIMA, S. Morphological changes contribute to apoptotic cell death and are affected by caspase-3 and caspase-6 inhibitors during Red sea bream iridovirus permissive replication. Virology 2004; 322: 220-230

21. KIBENGE, F. S. B., MUNIR, K., KIBENGE, M. T., JOSEPH, MONEKE. E. Infectious salmon anaemia virus: the causative agent, pathogenesis, and immunity. Animal Health Research Reviews. 2004; In press

22. KIBENGE, F. S. B., GARATE, O. N., JOHNSON, G., ARRIAGADA, R., KIBENGE, M. J. T. & WADOWSKA, D. Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Dis Aquat Org 2001; 45: 9-18

23. KIBENGE, F. S. B., LYAKU, J. R., RAINNIE, D. & HAMMELL, K. L. Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. J Gen Virol 2000; 81:143-150

24. LEIST, M. AND JAATTELA, M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001; 2: 589-598

25. LEWIS, J., WESSELINGH, S.L., GRIFFIN, D.E., HARDWICK, J.M. Sindbis virus induced-apoptosis in mouse brain correlates with neurovirulence. J Virol. 1996; 70: 1828-1835

26. LOWY, J. R. Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms. Int Rev Immunol 2003; 22: 425-449

27. MONEKE, E. E., KIBENGE, M. J. T., GROMAN, D., JOHNSON, G. R., IKEDE, B. O. & KIBENGE, F. S. B. Infectious salmon anaemia virus RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with infectious salmon anaemia virus. J Vet Diagn Invest 2003; 15: 407-417

28. NICHOLSON, D. W. Caspase structure, proteolytic substrates, and functions during apoptotic cell death. Cell Death Differ 1999; 6: 1028-1042

29. O'BRIEN, V. Viruses and apoptosis. J Gen Virol 1998; 79: 1833-1845

30. ROLLAND, J. B., BOUCHARD, D. A. & WINTON, J. R. The ASK cell line: An improved diagnostic tool for isolation, propagation and titration of the infectious salmon anaemia virus (ISAV). In: Miller, Otis, Cipriano, Rocco C, tech. Coords. International response to infectious salmon anaemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech Bull. 1902. Washington, DC: U.S Department of agriculture, Animal and Plant Health Inspection service, U.S Department of Interior, U.S. Geological Survey; U.S. Department of commerce, National Marine Fisheries Service: 2002; pp 63-68

31. ROULSTON, A., MARCELLUS, R. C. AND BRANTON, P. E. Viruses and apoptosis. Annu Rev Microbiol. 1999; 53: 577-628

32. SANCHEZ, L., ABUIN, M. & AMARO, R. Cytogenetic characterization of the AS cell line derived from the Atlantic salmon (*Salmo salar* L.). Cytogenetics Cell Genetics 1993; 64, 35-38

33. SCAFFIDI, P., MISTELLI, T., & BIANDI, M. E. Release of chromatin protein HMGBI by necrotic cells triggers inflammation. Nature 2002; 418: 191-195

34. THORNBERRY, N. A. AND LAZEBNIK, Y. Caspases: enemies within. Science 1998; 281: 1312-1316

35. VAUX, D. L. AND STRASSER, A. The molecular biology of apoptosis. Proc Natl Acad Sci USA 1996; 93: 2239-2244

36. WATANABE, M., HITOMI, M., VAN DER WEE, K., ROTHENBERG, F., FISHER, S. A., ZUCKER, R., SVOBODA, K. K. H., GOLDSMITH, E. C., HEISKANEN, K. M. & NIEMINEN, A. L. The pros and cons of apoptosis assays in the study of cells, tissues, and organs. Microsc. Microanal 2002; **8**: 375-391

37. WERGELAND, H. I. AND JAKOBSEN, R. A. A salmonid cell line (TO) for the production of infectious salmon anaemia virus (ISAV). Dis Aquat Org 2001; 44: 183-190

38. WHITE, E. Life, death, and the pursuit of apoptosis. Genes Dev 1996; 10: 1-15

39. ZHIRNOV, O. P., KSENOFONTOV, A. L., KUZMINA, S.G. AND KLENK, H. D. Interaction of influenza A virus M1 matrix protein with caspases. Biochemistry (Moscow) 2002a; 67: 534-539

40. ZHIRNOV, O. P., KONAKOVA, T. E., WOLFF, T. AND KLENK, H. D. NS1 Protein of Influenza A virus down-regulates apoptosis. J Virol 2002b; 76: 1617-1625

Chapter 4

Antibody-mediated growth of infectious salmon anaemia virus (ISAV) in macrophage-like fish cell lines*

* **Joseph, T.**, Kibenge, M.J.T., and Kibenge, F.S.B. 2003. Antibody-mediated growth of infectious salmon anaemia virus in macrophage-like fish cell lines. *Journal of General Virology*. 84:1701-1710

4.1. Summary

Protective immune response to ISAV has been previously demonstrated in experimentally infected fish as well as in vaccination trials, however, it does not provide full protection against the disease. There is limited information on how ISAV interacts with host defences, an issue to be considered when developing potential vaccination strategies. To study ISAV-antibody interactions, virus neutralization (VN) was performed on CHSE-214, SHK-1, and TO cell lines using three strains of ISAV and rabbit or fish anti-ISAV sera. Homologous VN titres of >1:1280 on CHSE-214 cells corresponded to only ≤1:80 on macrophage-like fish cell lines SHK-1 and TO despite using 1,000 and 2,000 times less virus, respectively. A similar assay using rabbit antiserum to infectious pancreatic necrosis virus (IPNV) provided a VN titre of 1:10,260 against IPNV on both CHSE-214 and TO cells. The poor neutralization of ISAV on TO cells was attributed to a possible Fc receptor mediated virus infectivity because: (1) neutralization by rabbit antiserum to ISAV was increased 48-fold in the presence of staphylococcal Protein A, and (2) using FITC-labeled virus and spectrofluorometry, a significant increase (p-value = 0.018) in fluorescence intensity of intracellular virus could be observed in assays of virus-antiserum mixtures in the absence of Protein A, compared to assays in the presence of Protein A. Neutralization of ISAV with fish antisera was observed only in CHSE-214 cells as Protein A could not restore the neutralization on TO cells. These findings demonstrate, for the first time, antibody-mediated infection of macrophage-like fish cell lines by ISAV, and thus have implications for ISA pathogenesis and vaccination as the virus in Atlantic salmon targets leucocytes, endothelial cells, and macrophages.

4.2. Introduction

Importance of humoral factors in the immune response to ISAV was demonstrated by passive immunization studies (Falk et al., 1995). Experimental infections demonstrated an immune response in Atlantic salmon against ISAV. However, this immune response does not provide full protection against the disease, as high mortality has usually been observed in both naturally and experimentally infected fish (Thorud 1991; Dannevig et al., 1993; Falk et al., 1995) and farmed Atlantic salmon that recover from clinical ISA continue to shed virus for long periods of time. Detection of ISAV in carrier fish required the use of reverse transcription-polymerase chain reaction (RT-PCR) (Devold et al., 2000), suggesting that the viral RNA is not abundant. However, such fish can transmit ISA to healthy Atlantic salmon upon co-habitation (Nylund et al., 1997).

The mechanisms of viral persistence and pathogenesis in ISAV infection at the cellular level are not well understood. The phenomenon of persistent viral infection in animals is unusual for orthomyxoviruses. There is no evidence of persistence of influenza virus genetic material in any mammalian or avian species. Influenza viruses are cleared from ducks in approximately seven days (Webster, 1999) although they can persist in carrier cultures (Urabe et al., 1993). In Atlantic salmon, ISAV seems to target leucocytic cells (Falk et al., 1995) and endothelial cells (Falk & Dannevig, 1995) or endothelial-associated cells (Falk et al., 2001), and macrophages (Moneke et al., 2003). *In-vitro*, ISAV readily replicates with production of CPE in SHK-1 cells (Dannevig et al., 1995), TO cells (Wergeland & Jakobsen, 2001), and ASK-2 cells (Rolland et al., 2002) which are macrophage-like cell lines

(Dannevig et al., 1997; Wegerland & Jakobsen, 2001; Rolland et al., 2002). Some strains of ISAV can also replicate and cause CPE in the CHSE -214 cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001).

It is known that macrophages of mammals and birds contain Fc receptors which enable internalization and digestion of virus particles coated with antibody (Mantovani et al., 1972). Antibody-enhanced infection occurs when monocytes and macrophages are more efficiently infected by virus-antibody complexes via Fc receptor-mediated endocytosis, than virus alone (Porterfeild, 1986). A number of human and animal viruses, belonging to at least 11 different virus families, have been shown to be capable of utilizing this mechanism for infection (Table 1.1). Many of these viruses demonstrating antibody-dependent enhancement *in-vitro* have also been associated with higher morbidity or mortality in cases of prior immunity (Sullivan et al., 2001). However, in the case of influenza virus, antibody-mediated internalization and growth of influenza A virus NWS (H1N1) has only been demonstrated in cultured murine macrophage-like cell line P388D1 in the presence of sub-neutralizing antiviral IgG (Ochiai et al., 1988). Although Fc receptors for fish IgM have been demonstrated on fish leukocytes (O'Dowd et al., 1998, Haynes et al., 1988), there is no report to date of Fc receptor-mediated infection of macrophages by fish viruses.

Preliminary virus neutralization studies on ISAV (Dr. Kibenge pers. comm.) using rabbit and fish polyclonal antisera to ISAV, suggested an antibody-mediated internalization of the virus depending on the cell line used. Total virus neutralization was observed in CHSE-214 cells,

whereas both rabbit and fish sera neutralized the virus to a lesser degree in SHK-1 and TO (macrophage-like cell lines). Therefore, it is hypothesized that fish macrophages are able to effectively internalize antibody-coated virus particles through Fc receptors as do mammalian macrophages, thereby allowing cellular penetration of antibody-coated ISAV and subsequent virus replication. This study investigated whether antiviral antibody enables the fish orthomyxovirus, ISAV, to infect the macrophage-like fish cell lines SHK-1 and TO.

4.3. Materials and methods

4.3.1. Viruses and cell cultures

Three different strains of ISAV, NBISA01, RPC-980-049(1), and RPC-990-002(4), were propagated in CHSE-214, SHK-1, and TO cell lines as described in section 2.1. Virus titration was performed in 24-hour old cell monolayers in 48-well plates and the virus titer in each cell line was determined from end point CPE by using the procedure described by Reed & Muench (1938). The virus titer was expressed as median tissue culture infectious dose ($TCID_{50}$) per 100 µl.

4.3.2. Polyclonal antibody preparations

Rabbit polyclonal antisera to purified ISAV isolates RPC-980-049(1) and RPC-990-002(4), the Atlantic salmon sera, the rainbow trout serum, and polyclonal rabbit anti-IPNV serum to IPNV strain FVX-8 used for the virus neutralization of IPNV were obtained as described previously (section 2.3). Normal rabbit serum, and sera from uninfected control rainbow trout and Atlantic salmon served as the negative control sera. All serum samples were heat-

inactivated at 56° C for 30 minutes prior to use in order to destroy complement activity. This treatment also served to inactivate any ISAV (Falk et al., 1997) that might have contaminated the fish sera from prior infection.

4.3.3. Virus neutralization (VN) assay

The VN tests were carried out on 24-hour old cell monolayers in 48-well cell culture plates as previously described (section 2.4). Cultures were examined microscopically for CPE to determine the VN test results after 10 days of incubation (14 days in case of the CHSE-214 cell line) at 16° C.

4.3.4. Blocking of Fc receptors

The staphylococcal Protein A (Sigma) used was resuspended to a concentration of 1 mg/ml in sterile distilled deionized water. Parallel VN assays were conducted on TO cell monolayers using ISAV strain NBISA01 and rabbit or Atlantic salmon anti-ISAV sera in the presence or absence of Protein A, as described by Olsen et al. (1992) with slight modifications. Briefly, Protein A was added to a final concentration of 200 µg/ml to the pre-incubated virus-antiserum mixtures which were further incubated for one hour at room temperature. The virus-antiserum mixtures were made in media without FBS in order to avoid competition for Protein A binding. The TO cell monolayers in 48-well cell culture plates were inoculated with the virus-antibody-Protein A mixtures and incubated for an additional one hour at room temperature. The residual virus-antibody complexes were then removed and fresh maintenance medium was added. The cultures were then incubated and

monitored as described for the VN assays above. Cells infected with virus in the presence or absence of Protein A served as controls.

4.3.5. Preparation of FITC-labeled ISAV

ISAV strain RPC-980-280-2 was propagated in TO cells and purified as described in section 2.5. Purified virus (600 µl) with a concentration of 0.5 mg viral protein/ml was reacted with an equal volume of 0.1 mg/ml fluoresceine isothiocyanate (FITC) dissolved in 0.5 M bicarbonate buffer (pH 9.5) for one hour at room temperature as previously described (Nichols et. al., 1992). Unconjugated dye was removed by passing the virus preparation through a Bio-Gel P-6DG (Bio Rad) column. The labeled virus was eluted using an equal volume of 1 x PBS and was then passed through a 0.45 µm syringe filter to eliminate virus aggregates. It was then stored at 4° C. For use, the labeled virus was divided into six equal volumes of 350 µl and each volume was used in the VN assay. A negative control for FITC labeled virus was prepared by replacing the purified virus with 1 x PBS in the labeling reaction.

4.3.6. Demonstration of antibody-mediated virus uptake using FITC-labeled ISAV

TO cells (1.4 x 10^5/ml) were grown in slide flasks (Fisher) (3 ml/flask) or six well tissue culture plates (Costar) (3 ml/well) overnight. Parallel VN assays were then set up using FITC-labeled ISAV, and rabbit anti-ISAV serum in presence and absence of staphylococcal Protein A. For this, 350 µl of FITC-labeled ISAV was incubated with equal amounts of 1:640 dilutions of the antiserum for one hour at room temperature. Preliminary experiments

established that Protein A when used at a concentration of 200 µg/ml could completely block the enhancing property of the rabbit anti-ISAV serum at a dilution of 1:640 in TO cells. Protein A was therefore, added to the virus-antibody mixture to a final concentration of 200 µg/ml and incubated for one hour at room temperature. The cell monolayers were then inoculated with the FITC-labeled ISAV-antiserum-protein A mixture and incubated for one hour at room temperature. TO cells inoculated with FITC-labeled ISAV alone were used as the positive control whereas TO cells inoculated with FITC and uninfected TO cells were used as the negative controls. All flasks and plates were incubated at 16° C for an additional three hours and were then analyzed using fluorescent microscopy or spectrofluorometry.

The slide flasks were processed for fluorescent microscopy by removing the inoculum from the flasks and washing the cell monolayers three times with 1 x PBS. The slides were then detached from the flasks and were fixed with 99% ethanol for 10 minutes at 4° C and air dried. The slides were analyzed using Fluoview 300 confocal laser scanning microscope (Olympus America Inc, USA) with a magnification of 400 x and Fluoview version 3.0 software.

The six well tissue culture plates were processed for spectrofluorometry by removing the inoculum from the tissue culture plate and washing the cell monolayers with 1 x PBS. Cells were detached using trypsin and resuspended in one millilitre of growth medium containing 10% FBS. Cells were pelleted at 500 x g for 10 minutes and resuspended in1 x PBS. Fifty microliters of cell suspension from each sample were placed in a single well of a Nunclon

F microwell plate and used to measure the fluorescence intensity using SpectraMAX Gemini XS spectrofluorometer (Molecular Divices Corporation, California, USA) at excitation of 485 nm and emission of 538 nm. Each sample was tested in five replicates. Results were analyzed by one way-ANOVA with Minitab version 13.

4.4. Results

4.4.1. Comparison of virus neutralization of ISAV on CHSE-214 cells and macrophage-like cells

In preliminary experiments of VN assays with rabbit antisera to ISAV using the selected fish cell lines, it was consistently observed that the VN antibody titres in the SHK-1 cell line were very low and did not accurately reflect the antigenic relationships between different ISAV strains. Virus neutralization by homologous antiserum could be demonstrated in CHSE-214 cells while the same virus-antibody mixtures showed little or no neutralization when tested in SHK-1 cells. Inoculation of SHK-1 cell monolayers with the virus-antibody mixtures resulted in CPE. Thus, meaningful virus neutralization data could not be obtained on the SHK-1 cell line. It was therefore decided to investigate whether macrophage-like fish cell lines are infected by antibody-bound ISAV, by setting up VN assays with both rabbit and fish polyclonal anti-ISAV antibodies using the same virus strains on three permissive fish cell lines as indicator systems.

The VN antibody titres obtained against three different ISAV strains on the three cell lines (CHSE-214, SHK-1, and TO) with four different ISAV antisera are summarized in Table 4.1.

The rabbit and Atlantic salmon antisera showed higher VN antibody titres on the CHSE-214 cell line than on SHK-1 and TO cell lines for the three different strains of ISAV used in the VN assay. In the most extreme case, rabbit anti-ISAV serum to ISAV strain RPC-990-002(4) had a homologous VN antibody titre of 1:4,800 on CHSE-214 cells but only 1:40 on SHK-1 cells and <1:10 on TO cells. With the Atlantic salmon and rainbow trout anti-ISAV sera, virus neutralization was observed only on CHSE-214 cells.

4.4.2. Titration of 100 $TCID_{50}$/100 µl of virus in different fish cell lines

The amount of virus used in the VN assay (100 $TCID_{50}$/100 µl) for each cell line was titrated on all three cell lines in order to rule out excessive amounts of virus as a cause of poor ISAV neutralization on SHK-1 and TO cells. Table 4.2 shows the results of these titrations. It is apparent that the 100 $TCID_{50}$ of the CHSE-214 cell system was 1,000 and 2,000 times more than that of the SHK-1 and TO cell systems, respectively. This is in contrast to the fact that the CHSE-214 cell system showed the highest VN antibody titres against ISAV. This indicates that the true VN antibody titres of the rabbit and fish anti-ISAV sera used were higher than those detected with the CHSE-214 cell system.

In the course of this work it was determined that the SHK-1 cell line at higher passages became less susceptible to ISAV. Since the pattern of neutralization of ISAV was the same for fish and rabbit antisera on both TO and SHK-1 cell lines (Table 4.1), it was decided to use only TO cells and CHSE-214 cells in further investigations.

Table 4.1. Virus neutralization (VN) using rabbit and fish anti-ISAV antisera on three different fish cell lines

Cell line and virus strain used in VN assay	Anti-ISAV serum[a] (rabbit) RPC-980-049(1)	(rabbit) RPC-990-002(4)	(Rainbow trout) NBISAV01	(Atlantic salmon) field serum
CHSE-214 cell line				
NBISAV01	960[b]	640	30	30
RPC-990-002(4)	2,560	4,800	40	40
RPC-980-049(1)	>1,280	480	120	30
SHK-1 cell line				
NBISAV01	80	20	<10	<10
RPC-990-002(4)	80	40	<10	<10
RPC-980-049(1)	40	20	<10	<10
TO cell line				
NBISAV01	160	20	< 40	<10
RPC-990-002(4)	1,280	<10	< 40	<10
RPC-980-049(1)	80	<10	< 40	<10

[a]Anti-ISAV serum raised in the laboratory to different strains of ISAV, except Atlantic salmon serum which was a pool of field serum samples from a natural ISA outbreak in Atlantic salmon. Animal species are listed in brackets.
[b]Virus neutralization titre, expressed as the highest dilution of serum to completely neutralize 100 $TCID_{50}$ of the respective ISAV strain.

Table 4.2. Titration of the 100 $TCID_{50}$ of ISAV strain NBISAV01 used in virus neutralization assays in three different fish cell lines

Cell line used in VN assay	Titration on cell line		
	CHSE-214	SHK-1	TO
CHSE-214	2.0[a]	5.0	5.3
SHK-1	<2.0	2.0	3.0
TO	<2.0	2.0	2.0

[a]Virus titres measured by end-point CPE and expressed as $\log_{10}$ $TCID_{50}$/100 µl.

4.4.3. Virus neutralization of IPNV in TO cells

In order to determine whether effective virus neutralization is possible on TO cells, VN of IPNV on TO cells was compared to that on CHSE-214 cells using rabbit anti-IPNV serum. The IPNV was neutralized to the same degree (VN titre of 1:10,260) on both CHSE-214 and TO cell lines. This demonstrated that the poor neutralization of ISAV on TO cells was unique to ISAV.

4.4.4.Virus neutralization of ISAV in TO cells in presence of staphylococcal Protein A

In order to test the hypothesis that the poor neutralization of ISAV by anti-ISAV sera on macrophage-like fish cell lines was due to Fc receptor-mediated uptake of antibody-coated virus particles by the cells, staphylococcal Protein A was used to block Fc receptors of the antibody prior to use in the VN assays. Appropriate controls of virus neutralizations in the absence of Protein A were also carried out. When Protein A was used in the VN assay with ISAV strain NBISA01 on TO cells, an antibody titre of 1:960 was obtained with rabbit anti-ISAV serum compared to a titre of 1:20 on TO cells without Protein A (Table 4.3). This was a 48-fold increase in ISAV VN titre on TO cells, and a 1.5-fold increase compared to the one obtained on CHSE-214 cells (Table 4.1). Protein A by itself did not adversely affect the infectivity of ISAV. This finding indicated that virus neutralization of ISAV on TO cells could be significantly increased by pre-treatment of the virus-antibody mixture with staphylococcal Protein A. Protein A did not however affect the VN titer on CHSE-214 cells (Table 4.3).

Table 4.3. Neutralization of ISAV strain NBISA01 in the presence of staphylococcal Protein A

Cell line	Anti-ISAV serum[a] (rabbit) RPC-990-002(4)	Anti-ISAV serum[a] (Atlantic salmon) NBISAV01
CHSE-214 cell line		
In the absence of Protein A	640[b]	60
In the presence of Protein A	640	60
TO cell line		
In the absence of Protein A	20	<10
In the presence of Protein A	960	<10

[a]Anti-ISAV serum raised in the laboratory to different strains of ISAV. Animal species are listed in brackets.

[b]Virus neutralization titre, expressed as the highest dilution of serum to completely neutralize 100 $TCID_{50}$ of ISAV strain NBISA01.

There was no virus neutralization observed when the rabbit anti-ISAV serum was used at dilutions of 1:40 to 1:320 on TO cells in the presence of Protein A, indicating that the binding effect of Protein A could be diluted out with higher antibody concentrations. In dilutions of 1:640 to 1:1280, Protein A could completely block the antibody-mediated uptake of virus. A similar biphasic response was also reported for FIP virus-antibody-macrophage interactions (Stoddart, 1989). Protein A could not restore the neutralization of ISAV by fish antisera on TO cells even at higher concentrations (Table 4.3). This may be due to the low affinity of Protein A to fish immunoglobulins (Bromage et al., 2004).

4.4.5. Demonstration of Fc receptor-mediated uptake of ISAV

In order to demonstrate that the ISAV uptake by macrophage-like cells in the presence of anti-ISAV serum was mediated by Fc receptors, the virus was purified and labelled with FITC, and intracellular virus was detected by two different methods, confocal laser scanning microscopy and spectrofluorometry. After inoculation, TO cells were incubated with FITC-labelled ISAV for four hours to allow the entry of the virus into the cells (Eliassen et al., 2000). Confocal laser scanning microscopy showed an apparent increase in the fluorescence of TO cells infected with ISAV in the presence of rabbit anti-ISAV serum (Fig. 4. 1b) as compared to TO cells infected with virus alone (Fig. 4. 1a). The fluorescence was reduced when the experiment was carried out in the presence of Protein A (Fig. 4. 1c). No fluorescence was observed in uninfected TO cells (Fig. 4. 1d). Analysis of serial optical sections of the TO cell monolayer infected with labeled ISAV and rabbit anti-ISAV serum (Fig. 4. 1b) with a depth increment of 0.5 μm showed maximum fluorescence between 6 μm

and 9 μm (Fig. 4. 2c and d). Because the thickness of cell membrane is only 7-10 nm, these data suggested that most of the fluorescence was intracellular, i.e., most of the labelled virus entered the cells in the presence of virus specific antibodies.

Fluorescence of intracellular virus was also detected by spectrofluorometry. Analysis was performed four hours after the VN assays using FITC-labelled ISAV. For this, all the cells were detached from each well of a six well plate and resuspended in 1 x PBS in order to measure fluorescence intensity. Significant reduction in the fluorescence intensity was observed in TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum and staphylococcal Protein A (Fig.4. 3. S1) as compared to TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig. 4. 3. S2) (p-value = 0.018) or TO cells infected with FITC-labelled ISAV alone (Fig. 4. 3. S3) (p-value = 0.0027). The difference observed between the fluorescence intensity of TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig.4.3. S2) and TO cells infected with FITC-labelled ISAV alone (Fig.4.3. S3) was not statistically significant (p-value = 0.8190). Uninfected TO cells (Fig.4.3. C1) (negative control) and TO cells inoculated with FITC (Fig.4.3. C2) (negative control) did not show any significant level of fluorescence. Viral entry into TO cells in presence of the rabbit anti-ISAV serum was confirmed by fluorescent microscopy (Fig. 4.4). In this experiment, virus neutralization observed in the presence of Protein A is consistent with the results obtained in the VN assays and confocal microscopic analysis.

Figure 4.1. Detection of intracellular virus by confocal laser scanning microscopy in TO cells infected with FITC-labeled ISAV (400x): **(a)** TO cells infected with FITC-labelled ISAV. **(b)** TO cells infected with FITC-labelled ISAV treated with 1:640 rabbit anti-ISAV serum. **(c)** TO cells infected with FITC-labelled ISAV treated with 1:640 rabbit anti-ISAV serum and staphylococcal Protein A. **(d)** Uninfected TO cells. An apparent increase in the fluorescence of TO cells infected with ISAV in the presence of rabbit anti-ISAV serum (b) was observed as compared to TO cells infected with virus alone (a) showing that ISAV was not neutralized by rabbit antiserum due to Fc receptor-mediated uptake of the antibody-coated virus. The fluorescence was reduced when the experiment was carried out in the presence of Protein A (c) due to the blocking of Fc receptor-mediated uptake of the virus. No fluorescence was observed in uninfected TO cells (d).

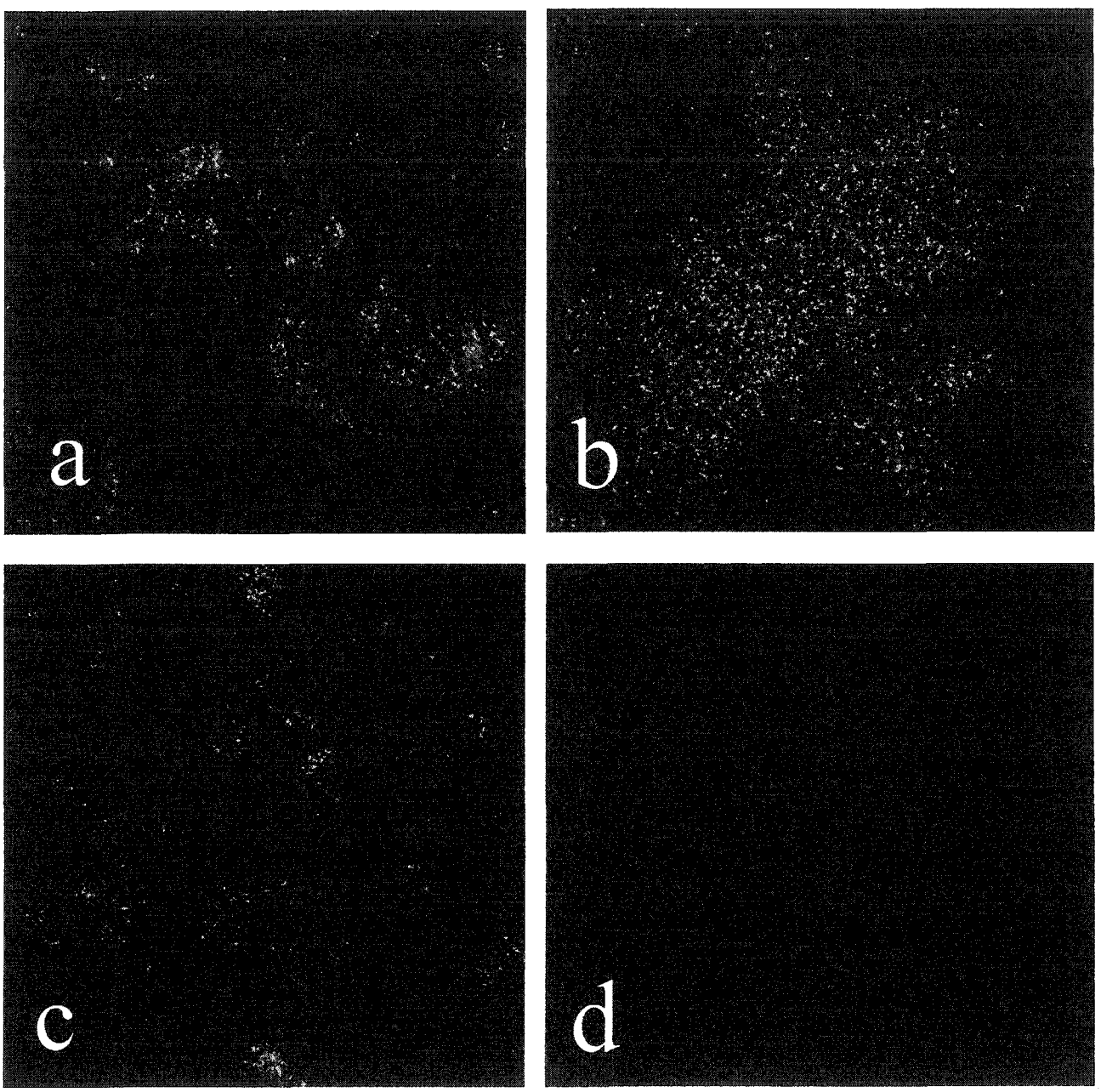


Fig. 4.1

Figure 4.2. Confocal laser scanning microscopic analysis of serial optical sections of the TO cell monolayer infected with labelled ISAV and rabbit anti-ISAV serum to ISAV strain RPC-990-002(4) (Fig.1b) with a depth increment of 0.5 μm (400x): **(a)** 0 μm, **(b)** 3 μm, **(c)** 6 μm, **(d)** 9 μm, **(e)** 12 μm, and **(f)** 15 μm depth. Maximum fluorescence was observed between 6 and 9 μm (c and d) suggesting that most of the fluorescence was intracellular since the thickness of the cell membrane is 7-10 nm.

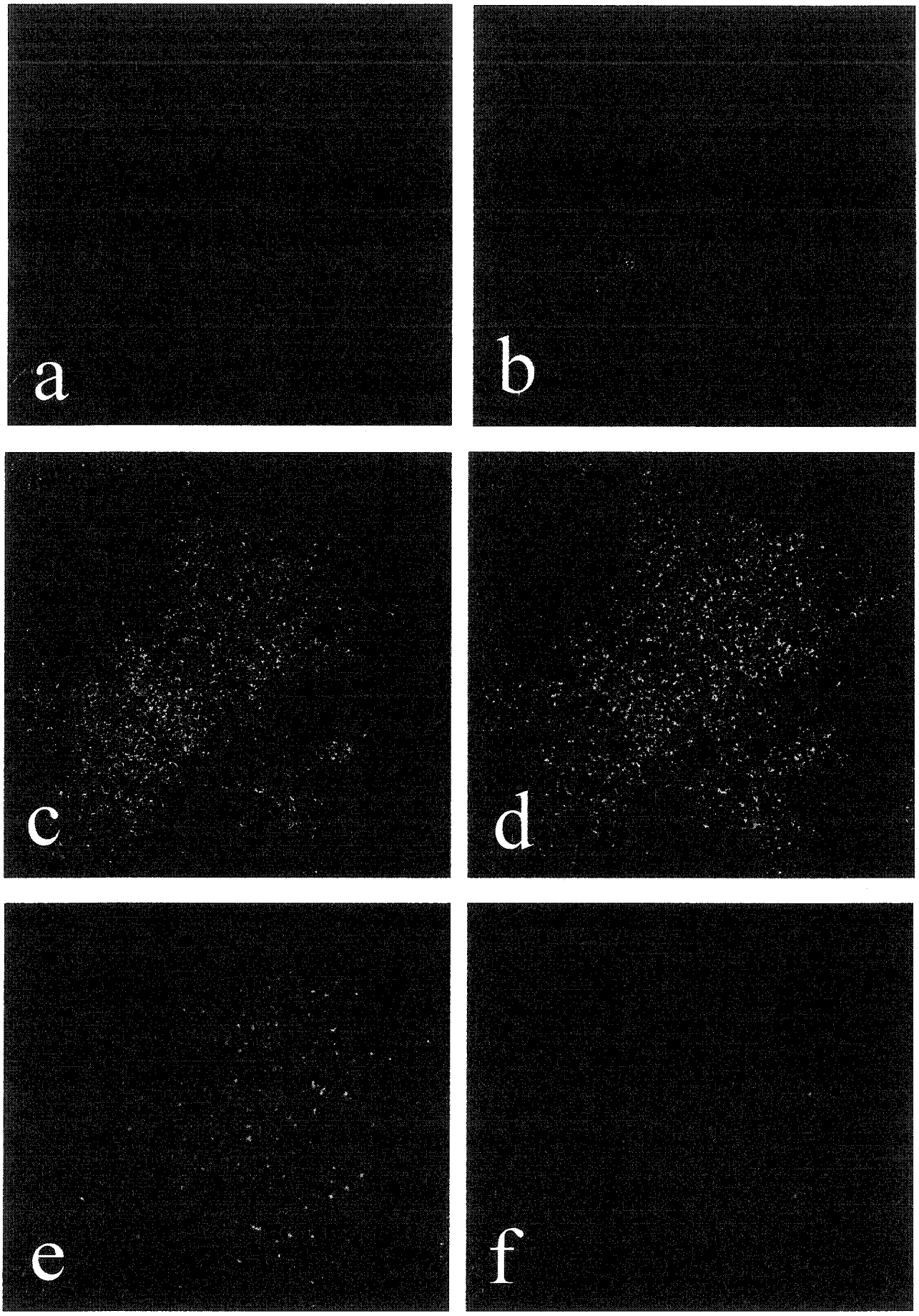


Fig. 4.2

Figure 4.3. Detection of intracellular virus by fluorescence microplate reader in TO cells infected with FITC-labelled ISAV (Excitation: 485nm, Emission: 538nm): **(C1)** Uninfected TO cells (negative control). **(C2)** TO cells inoculated with FITC (negative control). **(S1)** TO cells infected with FITC-labelled ISAV exposed to rabbit antiserum and staphylococcal Protein A in order to block the Fc receptor-mediated uptake of the virus. **(S2)** TO cells infected with FITC-labelled ISAV treated with rabbit antiserum. **(S3)** TO cells infected with FITC-labelled ISAV alone. Significant reduction in the fluorescence intensity was observed in (S1) as compared to (S2) (p-value = 0.018) and (S3) (p-value = 0.0027). No significant difference (p-value = 0.8190) was observed between the fluorescence intensity of (S2) and (S3).

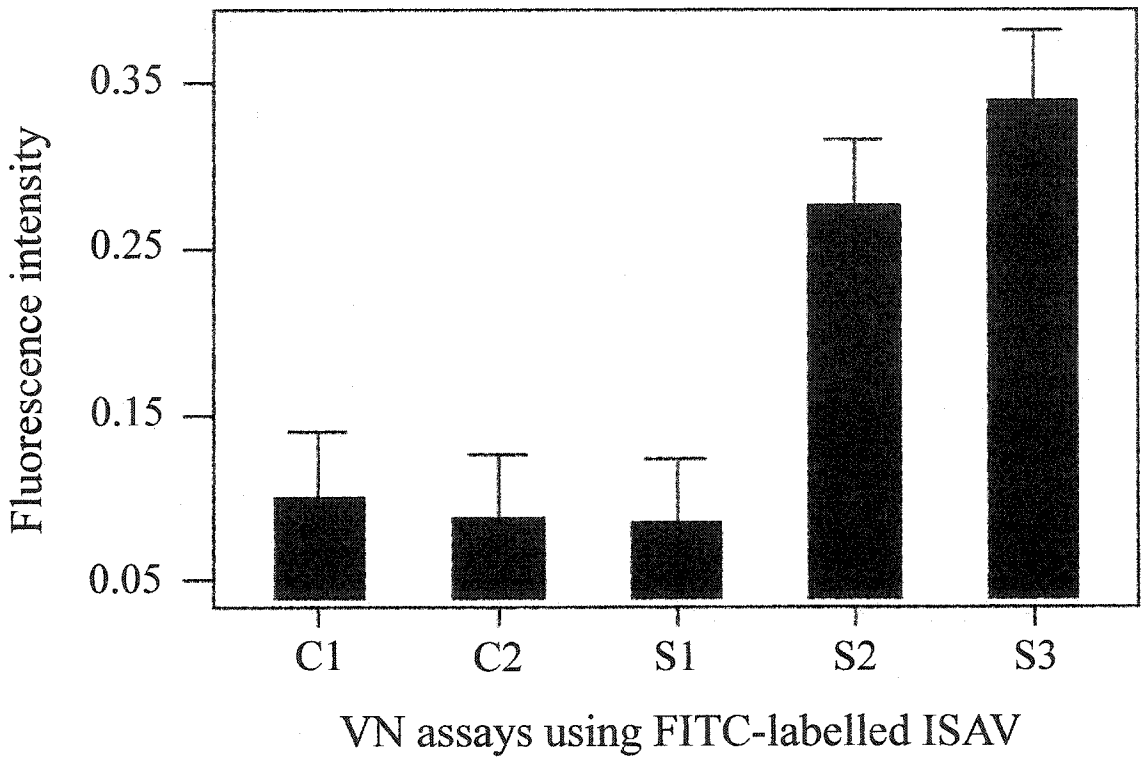


Fig. 4.3

Figure 4.4. TO cells showing intracellular fluorescence after four hours of infection with FITC-labelled ISAV in the presence of 1:640 dilution of rabbit anti-ISAV serum (630 x).

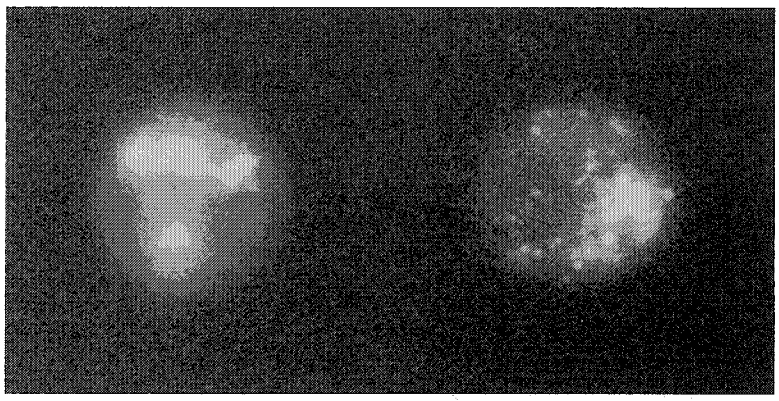


Fig. 4.4

4.5. Discussion

This study investigated whether antiviral antibodies enable the fish orthomyxovirus, ISAV, to infect macrophage-like fish cell lines SHK-1 and TO. Inoculation of SHK-1 and TO cell monolayers with the virus-antibody mixtures constantly resulted in CPE. Thus meaningful VN data are difficult to obtain in these cell lines. Falk et al. (1998) reported that a monoclonal antibody 3H6F8 against ISAV neutralized virus in SHK-1 cells. In that study, the neutralization was based on fluorescent antibody staining of the cells after incubation for 3 days and the exact endpoint for VN could not be determined (Falk et al., 1998). Moreover, the ability of a monoclonal antibody to neutralize virus does not rule out the possibility of polyclonal antiserum containing neutralizing and/or non-neutralizing antibodies that may mediate enhancement of virus infectivity.

In the present study, higher ISAV neutralizing antibody titres, as evidenced by complete absence of CPE, were obtained in the CHSE-214 cell system than in the SHK-1 and TO cell systems with the same antiserum. The following three experiments were used to show that the poor neutralization of ISAV on macrophage-like fish cell lines is due to antibody-mediated growth of the virus: (1) titration of 100 $TCID_{50}$/100 µl of virus in different fish cell lines showed that the CHSE-214 cell system used 1,000 and 2,000 times more virus than the SHK-1 and TO cell systems, respectively, but showed higher VN titres. This indicated that the true VN antibody titres of the antisera used are higher than the titres obtained on CHSE-214 cells; (2) anti-IPNV serum neutralized IPNV to the same degree in both CHSE-214 and TO cells, indicating that the poor neutralization of ISAV on TO cells was unique to ISAV;

and (3) virus neutralization of ISAV on TO cells was significantly increased in the presence of staphylococcal Protein A. Since Protein A is known to bind the Fc moiety of immunoglobulins (Olsen et al., 1992, Stoddart 1989), the data support the conclusion that rabbit immunoglobulins could bind to receptors on TO cells (possibly Fc receptors) and that the poor virus neutralization of ISAV in TO and SHK-1 cells may be due to Fc receptor-mediated uptake and subsequent replication of ISAV in these macrophage-like fish cell lines.

FITC-labelled ISAV, confocal laser scanning microscopy (Figs 4.1 and 4.2), and spectrofluorometry (Figs 4.3 and 4.4) were then used to show that antibody-mediated entry of the virus into TO cells may occur via Fc receptors. Confocal laser scanning microscopy showed an apparent increase in the fluorescence of TO cells infected with ISAV in the presence of rabbit anti-ISAV serum (Fig. 4. 1b) as compared to TO cells infected with virus alone (Fig. 4. 1a), i.e., antiviral antibodies facilitated the entry of virus into the cells. The fluorescence was reduced when the experiment was carried out in the presence of Protein A (Fig. 4. 1c), i.e., antibody-mediated uptake of the virus was blocked by Protein A, thereby neutralizing the virus in macrophage-like fish cells.

In spectrofluorometry, significant reduction in the fluorescence intensity was observed in TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum and staphylococcal Protein A (Fig.4. 3. S1) as compared to TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig. 4. 3. S2) (p-value = 0.018) or TO cells infected with FITC-labelled ISAV alone (Fig. 4. 3. S3) (p-value

= 0.0027). The difference observed between the fluorescence intensity of TO cells infected with FITC-labelled ISAV treated with rabbit anti-ISAV serum in the absence of Protein A (Fig.4.3. S2) and TO cells infected with FITC-labelled ISAV alone (Fig.4.3. S3) was not statistically significant (p-value = 0.8190). This observation further demonstrated that the use of Protein A blocked the putative Fc receptor-mediated uptake of antibody-coated ISAV and allowed virus neutralization by rabbit anti-ISAV serum.The two proposed mechanisms by which ISAV might infect fish macrophages, depending on the presence or absence of antiviral antibody, are depicted in Figure 4.5.

Virus neutralization assay was used previously on ISAV in TO cells with rabbit anti-ISAV sera to study the antigenic relationships of ISAV isolates (Kibenge et al., 2001b). In that study, the VN antibody titres obtained were sufficient to group the ISAV isolates into two main haemagglutinin subtypes. The TO cells were ideal for that study because all known ISAV isolates replicate and produce clearly discernible CPE in this cell line. In the present study, higher VN antibody titres were obtained in the VN assay with the CHSE-214 cell system whose 100 $TCID_{50}$ had 2,000 times more virus than that of the TO cell system. The CHSE-214 cell line, however, also has the disadvantage of not showing CPE when inoculated with some ISAV isolates (for example the ISAV isolates from Norway and Scotland) (Kibenge et al., 2000), and the low virus yields of CPE-positive ISAV isolates in this cell line suggest that it might not be sensitive enough to detect poorly neutralized virus.

Figure 4.5. Proposed mechanisms by which ISAV may infect fish macrophages. **(a)** Virus attachment followed by virus receptor-mediated endocytosis in all permissive cells (Eliassen et al., 2000). **(b)** Virus bound by specific antibody followed by Fc receptor-mediated endocytosis in fish macrophages. **(c)** Virus neutralization in macrophage-like cell line TO in the presence of staphylococcal Protein A.

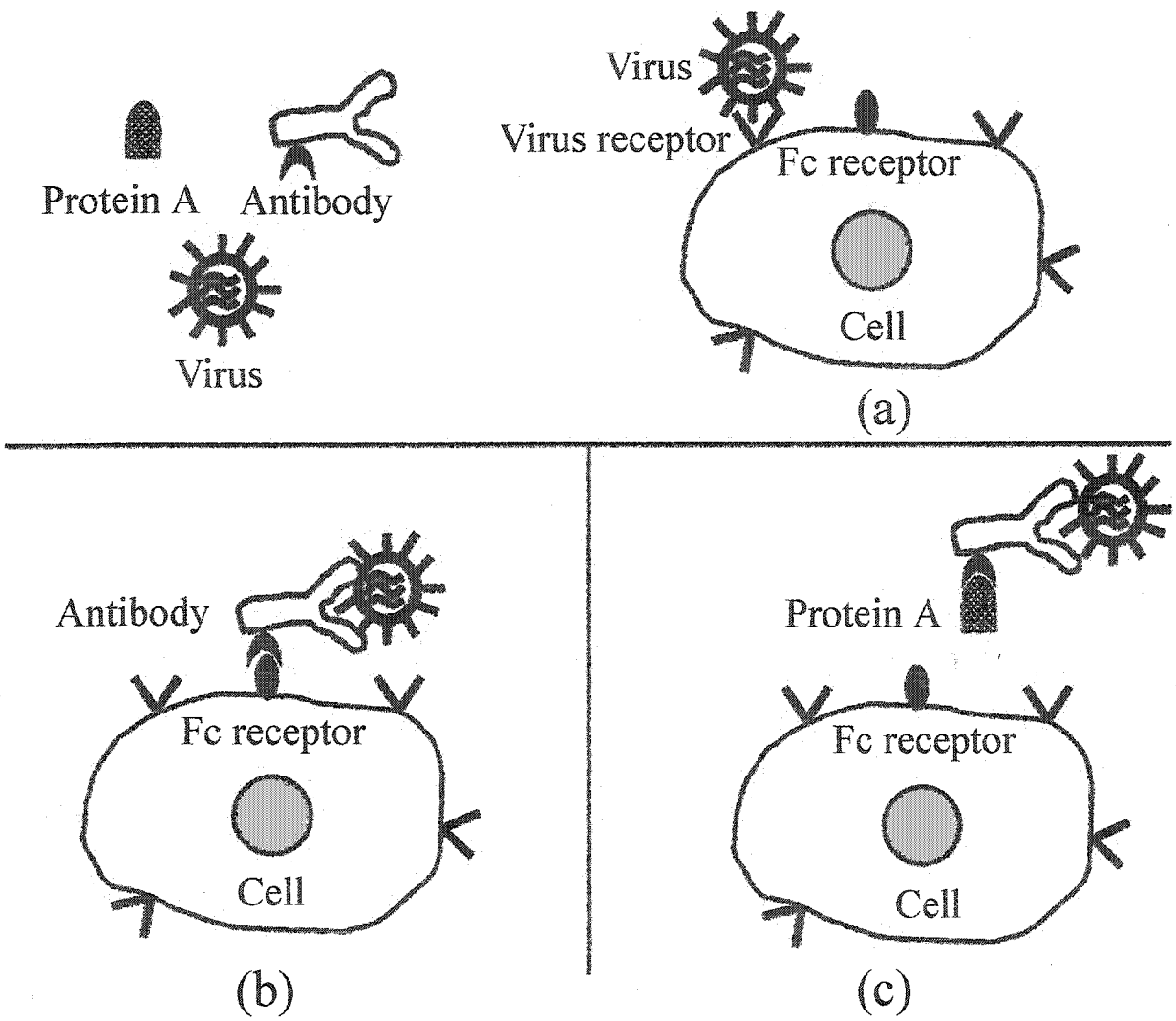


Fig. 4.5

All three strains of ISAV used in this study [NBISAV01, RPC-980-049(1), and RPC-990-002(4)] were cytopathic in the three fish cell lines (CHSE-214, SHK-1, and TO), and therefore the VN assays were easy to read microscopically.

Antibody-mediated growth of viruses is a well described phenomenon in mammals and birds (Sullivan et al., 2001). However, this is the first report of antibody-dependent and possibly Fc receptor-mediated, infection of fish cells by a fish virus. This phenomenon was demonstrated with rabbit anti-ISAV sera in TO cells suggesting that this macrophage-like fish cell line may have Fc receptors for mammalian immunoglobulins. The possibility of this is supported by the fact that a mammalian homologue of FcεRIγ (Fc receptor for IgE) chain exists in carp (Fujiki et al., 2000). In mammals, the existence of specific Fc receptors for five classes of immunoglobulins (FcαR, FcγR, FcδR, FcεR, and FcμR) has been recognized (Gessner et al., 1998). This study has shown that other mammalian Fc receptor homologues may also exist in fish macrophages. Fc receptors for shark IgM have been demonstrated on shark leukocytes, and high levels of immune complex receptors, possibly Fc receptors, have also been demonstrated on Atlantic salmon leukocytes (O' Dowd et al., 1998; Haynes et al., 1988). A most recent study documented that teleost natural killer (NK)-like cells are armed with IgM via a putative FcμR that enables them to kill targets by ADCC mechanism (Shen et al., 2003). In addition, there are three Expressed Sequence Tag (EST) sequences in the GenBank database, derived from teleost species, including Atlantic salmon, that have sequence similarity to mammalian Fc receptors (GenBank Accession numbers BG936360, AU050054 and BM425153). These pieces of evidence strongly suggest that Fc receptors are

present in teleost fish. Although mammalian IgM and complement can also mediate antibody-dependent enhanced virus infectivity via macrophage C3 receptors (Cardosa et al., 1983), that mechanism was considered unlikely in the present study because we used heat-inactivated anti-ISAV sera and FBS.

Not even higher concentrations of Protein A could restore the neutralization of ISAV by fish antisera on TO cells (Table 4.3). This might be due to poor binding of Protein A to the immunoglobulins of Atlantic salmon (Bromage et al., 2004). An alternative hypothesis that could be derived from the data is that only a small proportion of ISAV is able to infect CHSE-214 cells, and that it is an epitope that permits virus entry into CHSE-214 cells that is primarily seen and neutralized by rabbit antiserum. However, since the pattern of neutralization of ISAV was the same for fish and rabbit antisera on the three cell lines (Table 4.1), it is considered that poor neutralization of ISAV by fish antiserum on TO and SHK-1 cells is also due to Fc-mediated enhancement of infection. It is also possible that macrophage-like fish cell lines have different types of receptors which allow binding of fish or mammalian immunoglobulins. A previous study in fish described antibody-enhanced infectivity of fish rhabdoviruses that did not appear to involve Fc or C3 receptors but an unknown mechanism (Clerx et al., 1978).

Several different approaches have been used in the past to conclusively demonstrate antibody-mediated internalization of viruses *in-vitro* via Fc receptors including blocking with monoclonal anti-Fc receptor IgG and its Fab fragment (Peiris et al., 1981), with heat-

aggregated IgG (Daughaday et al., 1981; Lewis et al., 1988) or by binding with staphylococcal Protein A before inoculation (Chanas et al., 1982). The high affinity of Protein A for the Fc moiety of the antibodies was elegantly exploited by Stoddart (1989) to demonstrate Fc receptor-mediated uptake of FIP virus. In those studies, it was observed that Protein A dramatically reduced the level of FIP virus infectivity in the presence of enhancing antibodies. This observation indicated that binding of Protein A to Fc moiety could block the attachment of antibody-coated virus to Fc receptors and inhibit the antibody-enhanced infection of the virus (Olsen et al., 1992). Thus staphylococcal Protein A can be used to demonstrate Fc-mediated uptake of viruses as has been done in the present study.

Since ISAV in Atlantic salmon seems to target leucocytic cells, endothelial cells or endothelial-associated cells, and macrophages, it seems reasonable to speculate that Fc-mediated antibody-dependent enhancement of ISAV infection may occur *in-vivo*. This would accelerate the disease process by efficiently and specifically delivering virus to the very target cells within which the virus replicates. Additionally, such an infection, if non-cytolytic, would facilitate viral persistence in fish infected with ISAV. Conceptually, such infection-enhancing antibodies may be more effective when neutralizing antibodies either are absent, such as following antigenic drift and shift of the virus, or are consumed by virus excess.

In conclusion, this study has demonstrated, for the first time, antibody-mediated uptake and replication of ISAV, in macrophage-like fish cell lines SHK-1 and TO. Blockage of this mode of virus infection using Protein A suggests that this is an Fc-mediated infection of

cells. Experiments with purified immunoglobulin Fc and Fab fragments are required to definitively prove that the antibody-mediated uptake of ISAV in fish macrophage-like cells demonstrated here is mediated by Fc receptors. Systematic analysis of ISAV proteins by using both neutralizing and non-neutralizing monoclonal antibodies will also be necessary to identify the ISAV epitopes responsible for this phenomenon. Studies are also necessary to test whether antibodies to ISAV could cause disease enhancement or not. Findings from these studies will help to develop ISAV vaccines that have substantial preventative value.

4.6. References

1. BOLS, N. C., GANASSIN, R. C., TOM, D. J. & LEE, L. E. Growth of fish cell lines in glutamine-free media. Cytotechnology. 1994; 16: 159-66

2. BOUCHARD, D., KELEHER, W., OPITZ, H. M., BLAKE, S., EDWARDS, K.C. & NICHOLSON, B. L. Isolation of infectious salmon anaemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Dis Aquat Org. 1999; 35:131-137

3. BROMAGE, E.S., YE, J., OWENS, L., KAATTARI, I.M., KAATTARI, S.L. Use of staphylococcal protein A in the analysis of teleost immunoglobulin structural diversity. Dev Comp Immunol. 2004; 28: 803-814

4. CARDOSA , M. J., PORTFIELD, J.S. AND GORDON, S. Complement receptor mediates enhanced flavivirus replication in macrophages. J Exp Med. 1983;158: 258-263

5. CHANAS, A. C., GOULD, E. A., CLEGG, J.C. AND VARMA, M. G. Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity, and independently inhibit haemagglutination or haemolysis. J Gen Virol. 1982; 58: 37-46

6. CLERX, J. P. M., HORXINEK, M.C. AND OSTERHAUS, A. D. M. E. Neutralization and enhancement of infectivity of non-salmonid fish rhabdoviruses by rabbit and pike immune sera. J Gen Virol. 1978; 40: 297-308

7. DANNEVIG, B. H., FALK.K, KROSGUD J. Leucocytes from Atlantic Salmon (*Salmo salar)* experimentally infected with ISA exhibit an impaired response to mitogens. J Fish Dis. 1993; 16: 351-359

8. DANNEVIG, B. H., FALK, K. & NAMORK, E. Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol. 1995; 76:1353-1359

9. DANNEVIG, B. H., BRUDESETH, B. E., GJøEN, T. Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 1997; 7: 213-226

10. DAUGHADAY, C. C., BRANDT, W. E., MCCOWN, J. M. AND RUSSELL, P. K. Evidence for two mechanisms of dengue virus infection of adherent human monocytes: trypsin-sensitive virus receptors and trypsin-resistant immune complex receptors. Infection and Immunity. 1981; 32: 469-473

11. DEVOLD, M., KROSSøY, B., ASPEHAUG, V. & NYLUND, A. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Dis Aquat Org. 2000; 40:9-18

12. ELIASSEN, T. M., FROYSTAD, M. K., DANNEVIG, B. H., JANKOWSKA, M., BRECH, A., FALK, K., ROMOREN, K. AND GJOEN, T. Initial events in infectious salmon anaemia virus infection: evidence for the requirement of a low-pH step. J Virol. 2000; 74: 218-27

13. FALK K, AND DANNEVIG B H, A: Demonstration of a protective immune response in infectious salmon anaemia (ISA) infected Atlantic salmon *Salmo salar*. Dis Aquat Org 1995; 21: 1-5

14. FALK, K. AND DANNEVIG, B. H. Demonstration of infectious salmon anaemia (ISA) viral antigens in cell cultures and tissue sections. Virus Res. 1995; 26: 499-504.

15. FALK, K., NAMORK, E. AND DANNEVIG, B. H. Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. Dis Aquat Org. 1998; 34: 77-85

16. FALK, K., PRESS, C. M., LANDSVERK, T. AND DANNEVIG, B. H. Spleen and kidney of Atlantic salmon (*Salmo salar* L.) show histochemical changes early in the course of experimentally induced infectious salmon anaemia (ISA). Vet Immunol Immunopath. 1995; 49: 115-126

17. FALK, K., JOHANSEN, M. B., FERGUSON, H. W., ASPEHAUG, V., KROSSøY, B., BIERING, E. AND POPPE, T. Immunohistochemical detection of infectious salmon anaemia virus (ISAV). Proceedings of European Association of Fish Pathologists, Dublin, September. 2001; 10-14

18. FUJIKI, K., SHIN, D., NAKAO, M. AND YANO, T. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. Fish Shellfish Immunol. 2000; 10: 229-242

19. GESSENR, J. E., HEIKEN, H., TAMM, A., AND SCHMIDT, R. E. The IgG Fc receptor family. Annals Haemat. 1998; 76: 231-248

20. GRIFFITHS, S., COOK, M., MALLORY, B. & RITCHIE, R. Characterization of ISAV proteins from cell culture. Dis Aquat Org. 2001; 45:19-24

21. HAYNES, L., FULLER, L. AND MCKINNEY, C. Fc receptor for shark IgM. Dev Comp Immunol. 1988; 12: 561-571

22. KIBENGE, F. S. B., LYAKU, J. R., RAINNIE, D. AND HAMMELL, K. L. Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. J Gen Virol. 2000; 81:143-150

23. KIBENGE, F. S. B., KIBENGE, M. J. T., MCKENNA, P. K., STOTHARD, P. Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. J Gen Virol. 2001; 82: 2869-2879

24. LEWIS, R. M., COSGRIFF, T. M., GRIFFIN, B. Y. Immune serum increases arenavirus replication in monocytes. J Gen Virol. 1988; 69: 1735-1739

25. MANTOVANI, B., RABINOVICH, M., NUSSENZWEIG, V. Phagocytosis of immune complexes by macrophages: Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). J Exp Med. 1972; 135: 780-792

26. MONEKE , E. E., KIBENGE, M. J. T., GROMAN, D., JOHNSON, G.R., IKEDE, B.O., KIBENGE, F. S. B: Demonstration of infectious salmon anaemia virus (ISAV) RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with ISAV. J Vet Diag Invest 2003; 15: 407-417

27. NICHOLS, J. E., MOCK, D. J. AND ROBERTS, N. J. Use of FITC-labeled influenza virus and flow cytometry to assess binding and internalization of virus by monocytes-macrophages and lymphocytes. Arch Virol. 1992;130: 441-445

28. NYLUND, A., KVENSETH, A. M., KROSSøY, B. AND HODNELAND, K. Replication of the infectious salmon anaemia virus (ISAV) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis. 1997; 20: 275-279

29. OCHIAI, H., KUROKAWA, M., HAYASHI, K. & NIWAYAMA, S. Antibody-mediated growth of influenza A NWS virus in macrophage-like cell line P388D1. J Virol; 1988; 62: 20-26

30. O' DOWD, A. M., ELLIS, A. E., AND SECOMBES, C. J. Binding of immune complexes to Atlantic salmon peripheral blood leucocytes. Dev Comp Immunol. 1998; 22: 439-448

31. OLSEN, C. W., CORAPI, W. V., NGICHABE, C. K., BAINES, J. D. AND SCOTT, F. W. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J Virol. 1992; 66: 956-965

32. PEIRIS, J. S., GORDON, S., UNKELESS, J. C. AND PORTERFIELD, J. S. Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. Nature. 1981; 289: 189-191

33. PORTERFEILD, J. S. Antibody-dependent enhancement of viral infectivity. Adv Virus Res. 1986; 31: 335-355

34. REED, L. J. AND MUENCH, H. A simple method of estimating fifty percent endpoints. Am J. Hyg1938; 27: 493-497

35. ROLLAND, J.B, BOUCHARD, D.A, WINTON, J.R. Improved diagnosis of infectious salmon anaemia virus by use of a new cell line derived from Atlantic salmon kidney tissue. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp63-68

36. SANCHEZ, L., ABUIN, M. AND AMARO, R. Cytogenetic characterization of the AS cell line derived from the Atlantic salmon (*Salmo salar* L.). Cytogene Cell Gene.1993; 64: 35-38

37. SHEN, L., STUGE, T.B., EVENHUIS, J.P., BENGTEN, E., WILSON, M., CHINCHAR, V.G., CLEM, L.W., AND MILLER, N.W. Channel catfish NK-like cells are armed with IgM via putative FcμR. Dev Comp Immunol. 2003; 699-714

38. SOMMER, A. I. AND MENNEN, S. Multiplication and haemadsorbing activity of infectious salmon anaemia virus in the established Atlantic salmon cell line. J Gen Virol. 1997; 78:1891-1895

39. STODDART, C. A. Ph.D thesis. Cornell University, Ithaca, NY. 1989

40. SULLIVAN, N. J. Antibody-mediated enhancement of viral disease. Curr Top Microbiol Immunol. 2001; 260: 145-69

41. THORUD, K. Infectious salmon anaemia. Ph.D. Thesis. Norvegian college of veterinary medicine, Oslo, Norway. 1991

42. THORUD, K. & DJUPVIK, H. O. Infectious salmon anaemia in Atlantic salmon (*Salmo salar* L). Bulletin of European Association of Fish Pathologists.1988; 8: 109-111

43. URABE, M. T., TANAKA, T., ODAGIRI, M. Persistence of viral genes in a variant of MDBK cell after productive replication of a mutant of influenza virus A/WSN. Arch Virol. 1993; 128: 97-110

44. WEBSTER, R. G. Influenza viruses *(Orthomyxoviridae).* In: Encyclopaedia of Virology,. Edited by A. Granoff & R. G. Webster. Academic Press, NY. 2nd edn, 1999; pp. 824-829

45. WERGELAND, H. I. & JAKOBSEN, R. A. A salmonid cell line (TO) for the production of infectious salmon anaemia virus (ISAV). Dis Aquat Org. 2001; 44: 183-190.

Chapter 5

Characterization of selected monoclonal antibodies to ISAV

5.1. Summary

The study described in Chapter 4 demonstrated an antibody dependent, possibly Fc-receptor mediated viral entry and replication of ISAV in macrophage-like fish cell lines. If so, it is important to identify epitope(s) that induce the production of such harmful antibodies. The present study characterized four selected mAbs specific to ISAV. All the mAbs reacted specifically with purified ISAV in indirect ELISA and with viral protein(s) in the cytoplasm of ISAV-infected TO cells in IFAT. Isotyping revealed that mAb 4A11 and 9A7 were IgG_1, mAb 2D3 was IgM, and mAb 2B3 was of mixed isotypes, IgG_{2b} and IgM. None of the mAbs reacted with ISAV in Western blot analysis. High concentrations of mAbs 2D3 and 4A11, representing IgM and IgG isotypes, respectively, neutralized ISAV strains NBISA01 and RPC-980-049(1) in TO cells. Lower concentrations of these mAbs however did not enhance the viral infection in macrophage-like fish cell line TO. This study also compared two different *in-vitro* mAb production systems and showed that the VectraCell bioreactor had a better mAb yield and was more cost-effective than the Integra CELLine1000 membrane flask.

5.2. Introduction

The ability of a virus to infect and replicate within the cells of the mononuclear phagocyte system can be a major factor in the pathogenesis of virus infection because mononuclear phagocytes can facilitate access of viruses to susceptible tissues and organs, thereby hastening the infection process. Macrophages are one of the main target cells of ISAV *in-vivo* (Moneke et al., 2003). ISAV readily replicates with production of CPE in SHK-1 cells (Dannevig et al., 1995), TO cells (Wergeland & Jakobsen 2001), and ASK-2 cells (Rolland et al., 2002) which are macrophage-like cell lines (Dannevig et al., 1997; Wegerland & Jakobsen 2001; Rolland et al., 2003) derived from Atlantic salmon pronephros. The paradoxical ability of antiviral antibody to enhance virus infection of Fc receptor-bearing cells of mononuclear phagocyte system has been demonstrated across a wide range of virus families (reviewed in section 1.2.3).

Previous studies showed that meaningful virus neutralization data are difficult to obtain in ISAV-permissive, macrophage-like fish cell lines TO and SHK-1 because of a possible Fc receptor mediated antibody-dependent uptake and growth of ISAV in macrophage-like fish cell lines (Chapter 4). These studies also showed that antibody-mediated uptake and replication of ISAV occurs in the presence of both fish and mammalian antibodies in macrophage-like fish cell lines. Since the target cells of ISAV in Atlantic salmon include the cells of the mononuclear phagocyte system it seems reasonable to speculate that Fc-mediated antibody-dependent enhancement of ISAV infection may occur *in-vivo*. In addition, ISAV demonstrates most of the features that are common to viruses showing ADE of infection

including their ability to (a) replicate in part, or exclusively in macrophages, (b) induce the production of large amounts of antibodies that poorly neutralize even homologous virus, and (c) cause persistent infection which are commonly characterized by viremia of long duration. Antigenic diversity among different virus isolates is also a common feature of these viruses, which renders them partially resistant to neutralization by antibodies raised against heterologous isolates (Falk et al., 1995; Nylund et al., 1997; Kibenge et al., 2003; Tirado and Yoon 2003). Therefore it is important to know which antibodies to ISAV can cause enhancement of infection in macrophage-like fish cell lines. This would identify the viral epitopes responsible which then need to be excluded from ISAV vaccines. Enhancement of virus infection can be studied using antibodies from various sources including polyclonal antisera, mAbs, and antibodies purified from antisera, with mAbs providing the most sensitive means of studying ADE (Halstead et al., 1984).

Monoclonal antibodies are widely used for the antigenic analysis of viral proteins. They provide information about the virus structure and the immunological defense mechanism against virus infection which could not otherwise be obtained (McCullough 1986). The discriminatory power of hybridoma antibodies make them ideal reagents for investigation of antigenic relationships between viral proteins. They can be used to study the antigenic topology of viral proteins, which is essential for developing sensitive diagnostic tests and formulating efficacious vaccines (Yewdell and Gerhard 1981; McCullough 1986). Studies using mAbs showed that ADE is dependent on the epitopes and is not solely the result of the concentration of the virus specific antibodies (Halstead et al., 1984). Therefore mAbs can be

used to identify the viral epitopes that induce enhancing antibodies as well.

A major limiting factor in understanding the structure and functions of ISAV proteins and their role in ISA pathogenesis is the apparent scarcity of mAbs targeting clinically relevant epitopes on different ISAV proteins. To date, there is only one report describing the preparation of one mAb (IgG_1 isotype) designated 3H6F8 against ISAV (Falk et al., 1998). Immunofluorescence staining showed that this mAb was directed against a cytoplasmic ISAV protein. This mAb showed a strong reaction with IFAT on ISAV infected cell cultures, and with ELISA using lysed-infected cell cultures as antigen, but failed to react with ISAV polypeptides in Western blots, under both reducing and non-reducing conditions. No reaction was observed on formalin-fixed paraffin embedded tissue sections from diseased fish as well (Falk et al., 1998). Monoclonal antibody 3H6F8 has been shown to be specific for ISAV haemagglutinin protein, based on its neutralizing and heamagglutination-inhibition activities, and reactivity to recombinant haemagglutinin protein expressed in baculovirus (Falk et al., 1998; Krossoy et al., 2001; Rimstad et al., 2001). The primary goal of the present study was to characterize a panel of mAbs specific to ISAV in search of those that might enhance infectivity of ISAV in macrophage-like fish cell lines.

5.3. Materials and Methods

5.3.1. Selection and propagation of hybridoma cells

Immunization of BALB/c mice with ISAV strain RPC/NB 980-049-1 and fusion of the splenocytes from immunized mice with SP2/0 BALB/c parental myeloma cells were

performed by Immuno-Precise Antibodies Ltd. Victoria, BC. One-step selection and cloning of the hybridomas was performed in Clonacell™-HY medium (Stemcell Technologies Inc., Vancouver, BC). Clones were picked 11 days post-fusion and resuspended in wells of 96-well tissue culture plates in 200 µl of D-MEM (Invitogen) medium containing 1% hypoxanthine/thymidine (HAT), 20% fetal bovine serum (FBS), 2 mM GlutaMax I, 1 mM sodium pyruvate, 50 µg/ml gentamycin, 1% OPI and 0.6 ng/ml IL-6. After 4 days, the supernatants were screened by indirect ELISA for antibody activity. Subsequently ELISA was repeated for selected mAbs using purified preparations (100 µg/ml). For ELISA, 96-well microtiter plates (Falcon Pro Bind Assay plates, VWR) were coated with purified ISAV or FBS proteins or uninfected ASK-2 cellular proteins (1 µg/per well) in 100 µl of 0.2 M sodium bicarbonate buffer, pH 9.6, and incubated over night at 4° C. The plates were washed three times with 1 x PBS containing 0.05% (v/v) Tween-20 (T-PBS). Vacant sites were blocked with 3% normal goat serum in T-PBS (100 µl/well) for one hour at room temperature. Blocking medium was removed and plates were washed with T-PBS as before. One hundred microliters of hybridoma culture supernatant was added to each well and incubated the plates at 37° C for one hour. After washing with T-PBS, 100 µl of secondary antibody was added and incubated at 37° C for one hour. Culture supernatants from negative clones were used as negative controls. For the initial screening, goat anti-mouse IgG whole molecule conjugated to alkaline phosphatase (AP) (Sigma) was used at a dilution of 1:30,000 in T-PBS containing 1% goat serum. For subsequent assays, ImmunoPure goat anti-mouse IgG Fc fragment specific peroxidase (HRPO) conjugate (Pierce Biotechnology, Inc) was used for IgG mAbs at a dilution of 1:25,000, and goat anti-mouse IgM (µ-chain specific) AP

conjugate (Sigma) was used for IgM mAbs at a dilution of 1:30,000 in T-PBS containing 1% goat serum. Plates were washed as before and substrates p-nitrophenyl phosphate (BioRad) was added for AP conjugates and TMB microwell peroxidase (BioFX laboratories, USA) substrate was added for HRPO conjugates. The plates with AP substrate were incubated at 37° C overnight and OD was read at 405 nm in an automated microtiter ELISA reader (Spectra Max 340). Plates with TMB peroxidase substrates were incubated at 37° C and the reaction was stopped after 20 minutes by adding 100 µl of 1 N HCl in each well and OD was read at 450 nm. The initial screening was done in one replicate, but ELISA for selected mAbs was done in triplicate using purified mAbs at a concentration of 100 µg/ml. OD readings of ISAV-positive mAbs were expressed as mean OD readings ± standard error of the mean after subtracting the mean OD readings of the negative control. Putative ISAV positive hybridomas were re-cloned by limited dilution cloning in 24-well and 12-well tissue culture plates to ensure monoclonality.

Selected hybridomas were grown using the VectraCell - single use bioreactor system (BioVectra-DCL, Charlottetown, PEI). The bioreactor consists of a gas-permeable bag for cultivating cells *in-vitro* (Stang et al., 1997). The bioreactor was seeded with 1L of GIBCO Hybridoma Serum Free Medium (SFM) containing 1 x 10^6 cells per ml at approximately 90% viability. The bag was incubated at 37°C in a humidified incubator with 5% CO_2 atmosphere. Cells were counted every two days, and the medium containing the mAb was harvested when the viability reached 9% or less (approximately 2.5 to 3 weeks). The hybridoma producing mAb 4A11 was also grown using the INTEGRA cell culture device

CELLine 1000 (INTEGRA Biosciences, Wallisellen, Switzerland). This device consists of a membrane-based two-chamber flask where a 15 ml cell compartment is separated from an upper 1000 ml nutrient compartment by a 10 kDa molecular weight cut off membrane (Trebak et al., 1999; Marx, 1998). For use, the flask was seeded with 15 ml of cells at a concentration of 3 x 10^6 cells/ml in fresh complete growth medium. The nutrient compartment contained 1L of growth medium without FBS (designated the nutrient medium). Three harvests were done over a two week period.

5.3.2. Determination of mAb isotypes

The class and subclass of the mAbs were determined using the ClonaCell instantCHECK One-Minute Isotyping kit (Stemcell Technologies Inc., Vancouver, BC) following the manufacturer's instructions.

5.3.3. Concentration, purification, and quantitation of mAbs

The tissue culture supernatants from either T-75 cm^2 flasks or VectraCell system, and the Integra CELLine 1000 membrane flask were simultaneously concentrated and purified by precipitation with PEG-8000 in potassium phosphate buffer, pH 7.0. The protein pellet was resuspended in 1 x PBS and dialyzed overnight in 80 x volume of 1 x PBS buffer with two buffer chamber changes at 4° C. The protein concentration of the dialyzed antibody solution was then estimated from an OD reading taken at 280 nm in a spectrophotometer (1 OD_{280nm} = 0.69 mg/ml for mouse immunoglobulin), and the purified mAb was aliquoted prior to storage at -20° C until used. The purity of the mAbs was monitored before and after each

precipitation step using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein bands were visualized by Coomassie blue R250.

5.3.4. Indirect fluorescent antibody test (IFAT)

One-day old monolayers of TO cells grown in slide flasks were infected with ISAV isolate NBISA01. Slides were harvested when CPE developed and IFAT was carried out as described in section 2.2. Purified mAbs (1 mg/ml) and anti-mouse IgG (whole molecule) FITC conjugate (1:32) were used as primary and secondary antibodies respectively. Culture supernatant from a negative clone and uninfected TO cells were used as negative controls. The slides were examined under a fluorescent microscope.

5.3.5. Western blotting

Purified and concentrated (by ultracentrifugation) ISAV were resolved on 12.5% discontinuous SDS-PAGE. Twelve and half microliters of purified virus preparation was mixed with 12.5 µl of SDS-sample treatment buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue and 4% 2-mercaptoethanol just before use) and heated at 100°C for five minutes to denature the proteins before loading on to the SDS-PAGE gels. Electrophoresis was carried out at a constant current of 30 mA until the Bromophenol Blue dye had run off the bottom of the gel. The gel was then visualized by Coomassie Blue staining. For Western blotting, the SDS-PAGE gel and nitrocellulose membrane were briefly soaked in transfer buffer (48 mM Tris base, 39 mM glycine 0.037 % SDS, 20% methanol). Proteins were then transferred from the gel to the membrane in the same buffer at 4° C by

using a trans blot apparatus (Bio-Rad) for two hours at 0.65 mA/cm^2 (Sambrook et al., 1998). The transfer of the proteins was verified by the presence of Bench Mark prestained protein ladder (Invitrogen, Life Technologies) on the membrane. The membrane was rinsed several times with 1 x TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). Western blot assay was carried out using Amplified AP Immun-Blot kit (BioRad) according to manufacturer's instructions. Briefly, the membrane was blocked with 5% non-fat dry milk in TBS at room temperature and then washed with 1 x TBS. For primary antibodies mAbs 2D3, 2B3, 4A11, and 9A7 were prepared in TTBS (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5) at a concentration of 100 µg/ml and rabbit anti-ISAV serum was used at 1:100 dilution. Primary antibodies were then reacted individually with the membranes for one hour at room temperature. After washing the membranes were reacted with biotin-streptavidin AP conjugate followed by signal detection by the colour development reagent provided by the manufacturer.

5.3.6. Assay for antibody dependent enhancement (ADE)

Monoclonal antibodies 2D3 and 4A11 were used as neat (1 mg/ml), and dilutions 1:10, 1:10^2, 1:10^3, and 1:10^4 for ADE assay. In the preliminary assays, 100 µl of 100 $TCID_{50}$ of ISAV strain NBISA01 was mixed with equal volume of mAb preparation and used to infect TO cell monolayer in one well of a 48-well cell culture plates (Nunc). Cell monolayers in three wells were infected for each dilution of the mAb. Tissue culture plates were incubated at room temperature for one hour before addition of 500 µl of fresh maintenance medium to each well. Controls were set up by infecting the cell monolayers with 100 µl of virus suspension

containing 100$TCID_{50}$ alone. Cell monolayers along with the culture medium were harvested for virus grown in the presence and absence of mAbs at 24, 48, and 72 hour post-infection. Total virus harvested from three wells for each sample was pooled and frozen at -80° C. The samples were thawed and virus titration was performed in monolayers of TO cells grown in 48-well plates. The virus titre in each cell line was then determined from end point CPE by using the procedure described by Reed & Muench (1938). In subsequent assays, 100 $TCID_{50}$/100µl preparations of ISAV strains NBISA01, RPC-980-049(1), and U5575-1 were grown in the presence and absence of mAbs 2D3 and 4A11. Total virus was harvested at 48 hours post- infection and processed as described before. The virus titre was determined and expressed as $TCID_{50}$ per 100 µl. The titer of the virus grown in the presence and absence of mAbs were compared using logistic regression dose-response models (Finney 1978) for cell growth with doses measured on logarithmic scale. The hypothesis of equality of $TCID_{50}$ values in two dose-response series was tested by a likelihood-ratio test, and was considered statistically significant at $p<=0.05$.

5.4. Results

5.4.1. Selection and propagation of hybridoma clones

From the initial 900 clones that were picked into 96-well plates, 65 putative ISAV-positive hybridomas were expanded into 24-well plates prior to storage in liquid nitrogen. In a second screening using purified virus, uninfected ASK-2 cell lysate, and FBS proteins, four clones designated 4A11, 9A7, 2D3, and 2B3 reacted exclusively with purified virus and were thus selected for further characterization. The ClonaCell instantCHECK One-Minute Isotyping

kit was used to identify the class and subclass of these mAbs using hybridoma culture supernatants. Monoclonal antibodies 4A11 and 9A7 were typed as IgG_1, mAb 2D3 typed as IgM, and mAb 2B3 was of mixed isotypes IgG_{2b} and IgM (Table 5.1).

For further characterization, selected hybridomas were expanded to 6-well tissue culture plates and then to T-75 cm^2 flasks in regular growth medium. Hybridoma clones producing mAb 4A11, 9A7, and 2D3 were propagated using the 1L VectraCell system (BioVectra-DCL). The hybridoma cells producing mAb 4A11 were also grown in the Integra CELLine 1000 membrane flask (INTEGRA Biosciences) in order to compare the efficiency of the two *in-vitro* mAb production systems. The VectraCell bioreactor had a better mAb yield and was more cost effective than the Integra CELLine1000 membrane flask (Table 5.2). Monoclonal antibody 2B3 was only grown in T-75 cm^2 flasks.

5.4.2. IFAT

The immunofluorescence staining patterns exhibited by the four mAbs on ISAV-infected cell cultures were investigated. All the mAbs exclusively stained virus antigen present in the cytoplasm of ISAV infected TO cells (Fig. 5.1). Monoclonal antibody 2D3 showed a strong reaction whereas the other three mAbs showed weak staining. Uninfected TO cell or virus-infected TO cells where culture supernatant from a negative clone was used as primary antibody did not show fluorescent staining (Fig. 5.1).

Table 5.1. Monoclonal antibody properties

mAb	Isotype	ISAV antigen ELISA[a]	IFAT	Western blot
4A11	IgG_1	0.792±0.03	ISAV +ve [b]	-ve
9A7	IgG_1	0.448±0.03	ISAV +ve	-ve
2D3	IgM	0.643±0.01	ISAV +ve	-ve
2B3	IgM + IgG_{2b}	0.646±0.15	ISAV +ve	-ve

[a] Mean OD readings±SE after subtracting the mean OD readings of negative controls
[b] +ve denotes positive; -ve denotes negative test results

Table 5.2. Comparison of two *in-vitro* mAb production systems using ISAV mAb 4A11

	Integra CELLine CL 1000[a]	VectraCell System[b]
Production time	13 days	24 days
Total media volume used	3.03 L	1.0 L
Total volume mAb harvest	30 ml	1.0 L
Total volume concentrated and purified mAb	15 ml	50 ml
Purified mAb concentration	4.81mg/ml	4.79mg/ml
Total purified mAb yield	72.15mg	239.50mg
Cost for system disposables and culture medium per mAb	Can$283.91	Can$293.71
Production cost of purified mAb	Can$3.93/mg	Can$1.23/mg

[a]INTEGRA Biosiences (Wallisellen, Switzerland)

[b]BIOVECTRA-DCL (Charlottetown, PEI, Canada)

Figure 5.1. Indirect fluorescent antibody test (IFAT). Panels **(a), (b), (c),** and **(d)** are ISAV-infected TO cells showing fluorescent staining following IFAT using 2D3, 4A11, 2B3, and 9A7 respectively. Panel **(e)** represents that seen with uninfected TO cells or infected TO cells in which mAbs were replaced with supernatant from a negative clone.

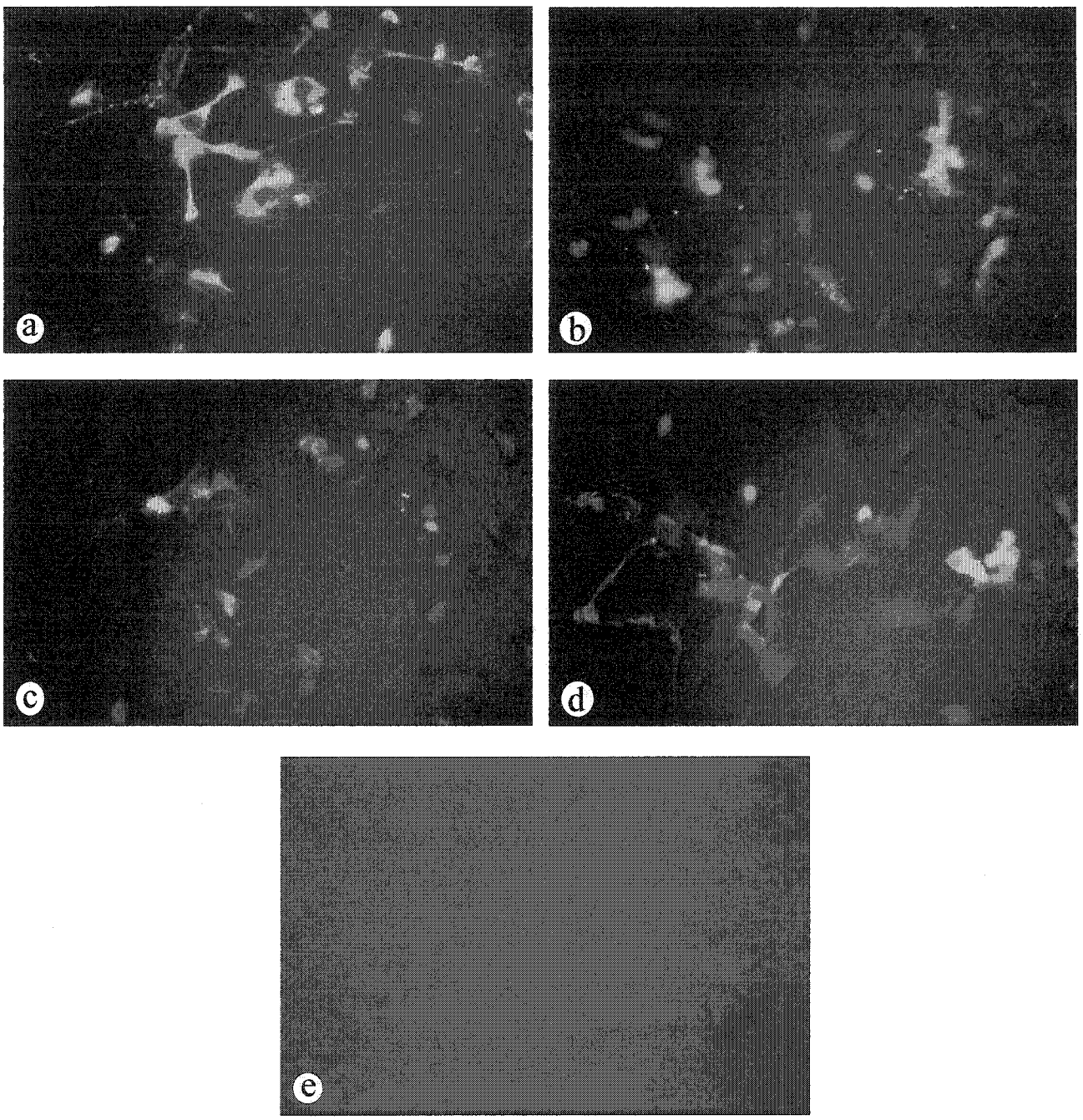


Fig. 5.1

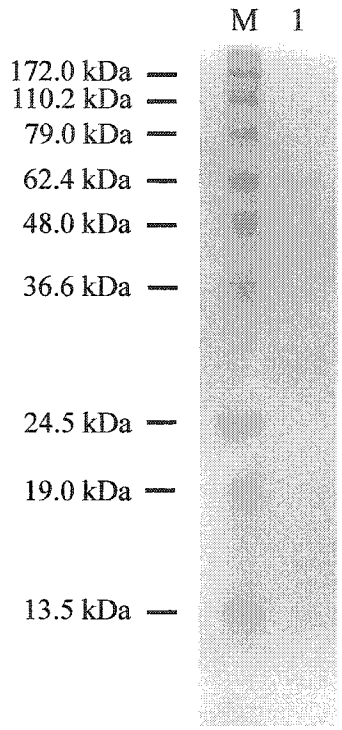
5.4.3. Western blotting

SDS-PAGE following Coomassie Blue staining clearly showed only one (74 kDa band) of the four major polypeptides of ISAV (Fig. 5. 2a). Western blot using rabbit anti-ISAV serum reacted however, with the four major polypeptides of ISAV (Fig. 5. 2b). Rabbit anti-ISAV serum also reacted with bovine serum albumin (BSA) which is a contaminant in the purified virus preparation. None of the mAbs reacted with ISAV proteins in Western blot analysis.

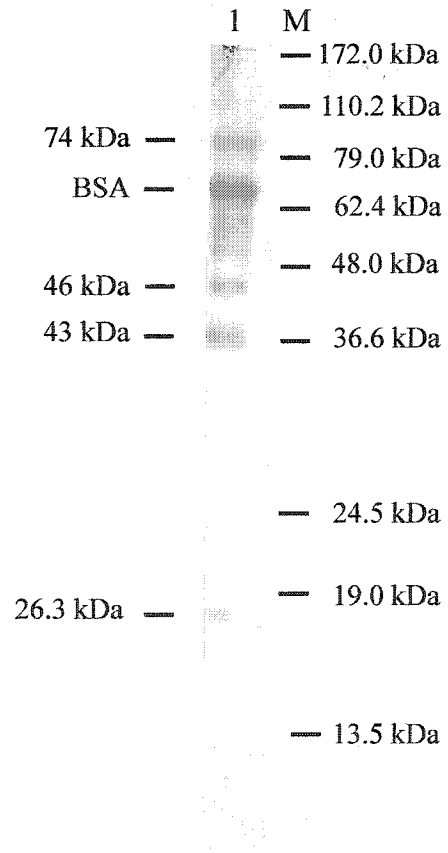
5.4.4 Assay for antibody dependent enhancement (ADE)

One IgG (4A11) and one IgM (2D3) mAbs were further selected for ADE assay. Preliminary assays using ISAV strain NBISA01 showed that samples harvested at 48 hours post-infection had sufficient virus for quantitation by titration. The titer of the virus grown in the presence of mAbs was compared with the titer of virus grown without mAbs in order to determine ADE. Statistical inference for the $TCID_{50}$ values were obtained using logistic regression dose-response models (Finney 1978) for cell growth with doses measured on logarithmic scale. No increase in virus production was observed when virus was grown in the presence of mAbs 2D3 and 4A11 (Tables 5. 3 and 5. 4). A significant reduction in the virus titer was observed when ISAV strains NBISA01 (p-value = 0.03) and RPC-980-049(1) (p-value = 0.00) were grown in the presence of mAb 2D3 (Table 5. 3) and RPC-980-049(1) (p-value = 0.00) was grown in the presence of mAb 4A11 (Table 5. 4) at a concentration of 1 mg/ml (neat). Harvesting of ISAV strain U5575-1 at 48 hour post-infection did not provide sufficient virus titer for statistical analysis.

Figure 5.2. SDS-PAGE and Western blot. **(a)** SDS-PAGE of purified ISAV after Coomassie blue staining. Lane M:Bench Mark prestained protein ladder (Invitrogen, Life Technologies), Lane 1: Purified ISAV. **(b)** Western blotting using rabbit anti-ISAV serum. Lane M: Bench Mark prestained protein ladder (Invitrogen, Life Technologies), Lane 1: Purified ISAV showing four major polypeptides and bovine serum albumin.



(a)



(b)

Fig. 5.2

Table 5.3. Titration of 100 $TCID_{50}$/100 µl of different strains of ISAV grown in TO cells in the presence of various dilutions of mAb 2D3 and harvested after 48 hours post-infection

		Virus titer				
		mAb dilution				
Virus strains	100 $TCID_{50}$ of the virus alone	Neat	1:10	$1:10^2$	$1:10^3$	$1:10^4$
NBISA01	4.16[a]	2.83 (0.03)[b]	3.83 (0.72)	3.5 (0.67)	3.83 (0.72)	3.5 (0.67)
RPC-980-049(1)	4.16	3.16 (0.00)	3.83 (0.20)	3.5 (0.07)	3.85 (0.20)	3.83 (0.20)
U5575-1	0.83	0.50	0.50	0.50	0.83	1.16

[a]Virus titres measured by end-point CPE and expressed as $\log_{10}$ $TCID_{50}$/100 µl

[b] p- Values are shown in the brackets

Table 5.4. Titration of 100 $TCID_{50}$/100 µl of different strains of ISAV grown in TO cells in the presence of various dilutions of mAb 4A11 and harvested after 48 hours post-infection

		Virus titer				
		mAb dilution				
Virus strains	100 $TCID_{50}$ of the virus alone	Neat	1:10	$1:10^2$	$1:10^3$	$1:10^4$
NBISA01	4.16[a]	3.16 (0.07)[b]	4.16 (0.86)	3.83 (0.72)	3.50 (0.28)	3.83 (0.59)
RPC-980-049(1)	4.16	2.83 (0.00)	3.83 (0.40)	3.50 (0.45)	3.83 (0.40)	4.16 (0.99)
U5575-1	0.83	0.50	1.16	0.50	0.50	0.50

[a] Virus titres measured by end-point CPE and expressed as $\log_{10}$ $TCID_{50}$/100 µl

[b] p- Values are shown in the brackets

5.5. Discussion

The main objective of this study was to characterize a panel of ISAV mAbs and use them to investigate whether antibodies to ISAV enhance infection in macrophage-like fish cell lines. In Chapter 4 it was demonstrated that both mammalian and fish antibodies can mediate uptake and replication of ISAV in macrophage-like fish cell lines thereby abrogating virus neutralization in these cell lines. In the present study, several hybridoma cell lines producing mAbs specific to different ISAV proteins were established. Isolation of hybridoma clones was carried out using one-step selection with Clonacell^{TM}-HY medium (Stemcell Technologies Inc., Vancouver, BC). This proprietary semi-solid medium has several advantages over standard hybridoma selection and cloning methods which allowed the isolation of over 900 hybridoma clones from one fusion. Four hybridoma clones were identified that reacted specifically with purified ISAV antigen in ELISA (Table 5.1).

In IFAT, mAb 2D3 showed a very strong reaction with ISAV protein in the cytoplasm of virus-infected TO cells (Fig. 5.1a) whereas mAbs 411, 9A7, and 2B3 reacted weakly (Fig. 5.1b,c, and d). None of the mAbs showed positive reaction in uninfected TO cells. Similarly culture supernatants from negative clones did not show positive reaction in ISAV infected TO cells in IFAT. This indicated that positive reactions showed by the mAbs in IFAT are specific to ISAV proteins. Since mAb 2D3 reacted very well with ISAV proteins both in ELISA and IFAT, it may be used for developing diagnostic assays.

None of the mAbs reacted with ISAV proteins in Western blot analysis. Therefore, it was not

possible to identify the viral proteins to which the mAbs were directed. In contrast, rabbit anti-ISAV serum reacted with four major structural proteins of ISAV in the same Western blot analysis. This showed that the assay was carried out properly and there was sufficient amount of viral proteins present in the nitrocellulose membrane. It is possible that all these mAbs are directed to conformational epitopes on ISAV proteins which are altered in the denaturing conditions of SDS-PAGE. Analysis using non-denaturing gel or 2D gel electrophoresis and Western blotting might help to determine the target protein(s) of these mAbs.

Based on their reactions in ELISA and IFAT, one IgG (mAb 4A11) and one IgM (mAb 2D3) were selected to investigate ADE of ISAV infection in TO cells. In the preliminary experiments $100TCID_{50}/100$ µl of ISAV strain NBISA01 was grown in the presence of various concentrations of the mAb and the total virus was harvested at different time points post- infection. Titration of these samples showed that samples harvested at 48 hours post-infection had sufficient virus for titration by end-point CPE in TO cells. Therefore, three different strains of ISAV, NBISA01, RPC-980-049(1), and U5575-1 were used in the subsequent assay and samples were harvested at 48 hours post-infection. Titration of the samples in TO cells showed that mAbs 4A11 and 2D3 did not cause enhancement of ISAV infection in TO cells. A significant reduction in the virus titer was observed when ISAV strains NBISA01 (p-value = 0.03) and RPC-980-049(1) (p-value = 0.00) were grown in the presence of mAb 2D3 (Table 5.3) and RPC-980-049(1) (p-value = 0.00) was grown in the presence of mAb 4A11 (Table 5.4) at a concentration of 1 mg/ml (neat). This observation

indicated that at this concentration these mAbs cause neutralization. At lower concentrations, no significant difference was observed between the titer of virus grown in the presence and absence of mAbs (Table 5.3 and 5.4). This suggested that at these concentrations, antibodies do not have any effect on virus replication in TO cells. No statistical analysis could be done in the case of a less virulent ISAV strain U5575-1 since the sample harvested at 48 hours post-infection did not provide sufficient virus titer for statistical analysis.

Morens and Halstead (1990) proposed the following criteria in order to verify true ADE, (i) a statistically significant increase in the virus production as measured by quantitative assays at different time points on the one step growth curve, (ii) serial dilution of the pre-incubated antibody source must produce an 'enhancement profile' or rising, peaking, and declining production of infectious virions over at least a 10^3- fold dilution range, (iii) the dilution of the antibody source at which ADE is maximal must be related to its reactivity in other serological tests that measure envelope binding, (iv) ADE should be detected with different antibody sources and virus strains tested over a range of multiplicity of infections, (v) other causes of increased virus production must be ruled out, including a stabilizing or stimulating effect of the antibody source on the infected cell, prevention of thermal decay of virus by antibody, and competition for cell receptors and antibodies by either non-infectious or soluble or membrane antigens in the virus stock. Therefore, further studies must be carried out using different mAbs and antisera from various sources in order to investigate ADE of ISAV infection.

This study also compared two high cell density *in-vitro* methods of producing mAbs, the VectraCell bioreactor to the well established Integra CELLine flask. Such *in-vitro* methods are mouse-friendly (unlike the *in-vivo* ascites production) with high mAb yields that are free of murine protein contamination. By far, the VectraCell system resulted in the most mAb production, yielding 293.71 mg for the 1L of medium used. The Integra CELLine1000 membrane flask has been reported to produce 66 mg of mAb in two weeks (Trebak et al., 1999). This is close to the 72.15 mg of mAb obtained with this system for the same duration in the present study. Thus the VectraCell bioreactor had a better mAb yield and was more cost effective than the Integra CELLine1000 membrane flask. Cells are grown in higher densities in Integra CELLine1000 membrane flasks. Therefore, significantly higher concentrations of mAb are obtained in a smaller volume, which minimizes downstream concentration steps.

In conclusion, four mAbs designated 4A11, 9A7, 2D3 and 2B3, were characterized. All the mAbs specifically reacted with purified ISAV in an indirect ELISA. The indirect immunofluorescence antibody test on ISAV-infected TO cells showed that, mAbs 2D3, 4A11, 9A7, and 2B3 target viral epitopes widely distributed in the cytoplasm. Western blot analysis showed that all the five mAbs are directed to conformational epitopes on ISAV protein(s). Monoclonal antibodies 2D3 and 4A11, representing IgM and IgG isotypes respectively, neutralized ISAV strain NBISA01 in TO cells when used at very high concentrations. Lower concentrations of these mAbs did not enhance the viral infection in macrophage-like fish cell line TO. Further experiments using mAbs targeting various

proteins of ISAV are required to identify proteins which mediate viral entry in macrophage-like fish cell lines.

5.6. References

1. DANNEVIG, B. H., FALK, K., AND PRESS, C. M.: Propagation of infectious salmon anaemia (ISA) virus in cell culture. Vet Res 1995; 26: 438-442

2. DANNEVIG, B.H., BRUDESETH, B.E., GJOEN, T., RODE, M., WERGELAND, H.I., EVENSEN, O., AND PRESS, C.M: Characterization of a long- term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 1997; 7:213-226

3. FALK K, AND DANNEVIG B H, A: Demonstration of a protective immune response in infectious salmon anaemia (ISA) infected Atlantic salmon *Salmo salar*. Dis Aquat Org 1995; 21: 1-5

4. FALK, K., NAMORK, E., AND DANNEVIG, B. H.: Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. Dis Aquat Org 1998; 34: 77-85

5. FINNEY, D. J. Statistical Methods in Biological Assay, 1978. 3rd ed. Charles Griffin and Co. Ltd., London.

6. HALSTEAD, S. B., VENKATESHAN, C. N., GENTRY, M. K., AND LARSEN, L. K.: Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. J. Immunol. 1984; 132: 1529-1532

7. KIBENGE, F.S.B., KIBENGE, M.T., JOSEPH, T., AND McDOUGALL, J. The development of vaccines in Canada in regards to ISAV. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp 39-49

8. KROSSOY, B., DEVOLD, M., SANDERS, L., PER MORTEN KNAPPSKOG: Cloning and identification of infectious salmon anemia virus haemagglutinin. J Gen Virol. 2001; 82: 1-9

9. MARX, U.: Membrane-based cell culture technologies: a scientifically and economically satisfactory alternative to malignant ascites production for monoclonal antibodies. Res Immunol. 1998,149(6):557-9

10. McCULLOUGH, K. C.: Monoclonal antibodies: implications for virology. Brief review. Arch. Virol.1986; 87: 1-36

11. MONEKE, E.E., KIBENGE, M.J.T., GROMAN, D., JOHNSON, G.R., IKEDE, B.O., KIBENGE, F.S.B: Demonstration of infectious salmon anaemia virus (ISAV) RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with ISAV. J Vet Diag Invest. 2003; 15: 407-417

12. MORENS, D. M., VENKATESHAN, C. N., AND HALSTEAD, S. B.: Dengue 4 virus monoclonal antibodies identify epitopes that mediate immune infection enhancement of dengue 2 viruses. J. Gen. Virol. 1987; 68: 91-98

13. NYLUND A: Reservoir species for the infectious salmon anemia virus (ISAV). Proceedings from workshop on Infectious salmon anemia, St Andrews, New Brunswick 29, 1997

14. REED, L. J. AND MUENCH, H. A simple method of estimating fifty percent endpoints. Am J Hyg1938; 27: 493-497

15. RIMSTAD, E., MJAALAND,S., SNOW, M., MIKALSEN, A.B., CUNNINGHAM, C.O: Characterization of the infectious Salmon Anemia Virus Genomic Segment That Encodes the Putaitve Haemagglutinin. J Virol. 2001; 75: 5352-5356

16. ROLLAND, J.B, BOUCHARD, D.A, AND WINTON, J.R: Improved diagnosis of infectious salmon anaemia virus by use of a new cell line derived from Atlantic salmon kidney tissue. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp63-68

17. SAMBROOK, J., Fritsch, E.F., AND MANIATIS, T. Molecular Cloning: A Laboratory Manual vol. 3, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.1989; pp. 18.62-18.67

18. STANG, B., WOOD, P.A., REDDINGTON, J.J., REDDINGTON, G.M., AND HEIDEL, J.R. Monoclonal antibody production in Gas-Permeable Bags using serum-free media. Proceedings of the John Hopkins Center of alternatives to animal testing, monoclonal

antibody workshop. September 24-25, 1997

19. TIRADO, S. M., YOON, K. J.: Antibody-dependent enhancement of virus infection and disease. Viral Immunol. 2003; 16: 69-86

20. TREBAK, M.,CHONG, J.M., HERLYN, D., AND SPEICHER, D.W.: Efficient laboratory-scale production of monoclonal antibodies using membrane-based high-density cell culture technology. Immunol Methods. 1999, 230(1-2):59-70.

21. WERGELAND, H. I., AND JAKOBSEN, R. A.: A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). Dis Aquat Org 2001; 44: 183-190

22. YEWDELL, J. W., AND GERHARD,W.: Antigenic characterization of viruses by monoclonal antibodies. Annu. Rev. Microbiol. 1981; 35: 185-206

Chapter 6

General discussion and future directions

6.1. General Discussion

The interactions that occur between viral proteins and host factors, such as cellular proteins and signal transduction machinery, have a significant influence on the replication, persistence, and pathogenesis of all viruses (Lyles 2000). ISAV causes overt and fatal systemic infection in farmed Atlantic salmon (Byrne et al., 1998; Dannevig et al., 1995a,b; Nylund et al., 1994, 1996; Rodger et al., 1998) and asymptomatic infection in feral fish (Raynard et al., 2001a). At the same time Atlantic salmon that recover from clinical ISA as well as subclinically infected trout and herring can transmit ISAV to healthy Atlantic salmon through cohabitation (Nylund and Jakobsen, 1995; Nylund et al., 1997, 2002; McAllister et al., 2003). The mechanisms of pathogenesis and persistence of ISAV have not been adequately studied at the molecular level. It is important to know how this virus interacts with the host cell, in order to develop effective control strategies. This study analyzed the host-pathogen interactions of ISAV *in-vitro* using fish cell lines that are derived from the very target cells for ISAV *in-vivo*.

It is important to understand the nature of a viral infection in the individual host animal in order to understand infection in the whole host population. Similarly, understanding of the nature of infection in the individual cell is key to understanding infection in complex tissues, organs, and whole host animals (Murphy et al., 1999). The clinical symptoms of viral disease in the host result primarily from the host response to infection. This response is initiated by cell injury caused by virus replication. Cell injury can result from the direct effect of virus replication on the cell or from the consequences of the host's innate and adaptive immune

response or from both (Murphy et al., 1999). Infection of cultured cells by many viruses results in visible changes in the cells collectively called CPE. Direct alteration of the cell by viral infection can clearly account for some of the changes observed during infection in animal host (Lyles 2000). Because virus-induced cytopathic effects are clearly relevant to viral pathogenesis, it is important to understand how viruses alter host cell structure and metabolism. Viruses can cause visible cell damage by apoptosis or necrosis.

The main purpose of this study was to investigate the mechanism of cell death during ISAV infection and the putative antibody mediated growth of ISAV in macrophage-like fish cell lines. In this study, permissive fish cell lines SHK-1, CHSE-214, and TO were used to determine if ISAV-induced CPE is due to apoptosis or necrosis (Chapter 3). Induction of apoptosis in SHK-1 and CHSE-214 cells were demonstrated by DNA fragmentation assay (Fig. 3. 2). Analysis of apoptosis induced by two strains of ISAV which differed in virulence showed that CPE in SHK-1 and CHSE-214 cells is caused by apoptosis and that cell type dependent induction of apoptosis is not virus strain specific. Apoptosis induced by ISAV in SHK-1 cells was also confirmed by fragment end labeling assay (Fig. 3.3). However TO cells did not show positive reaction either in DNA fragmentation or fragment end labeling assays. This indicated that ISAV may be inducing necrotic type of cell death in TO cells. Recent studies have identified a marker for necrosis namely HMGB 1 protein. This group of proteins is a chromatin binding factor which will be leaked out of the nucleus during necrosis but not in apoptosis. Release of HMGB1 protein results in the inflammatory reactions that follow necrotic type of cell death (Scaffidi et al., 2002). Indirect fluorescent antibody test using anti-

HMGB antibodies clearly showed the leakage of HMGB1 protein from the nucleus of virus infected TO cells but not from infected SHK-1 cell (Fig. 3.4 and Fig. 3.5). These results demonstrated that the mechanism of cell death during ISAV infection is cell type dependent.

The biochemical events involved in the replication of ISAV have not been fully explored but the replication strategy of the virus seems to resemble that of influenza viruses (Hovland et al., 1994; Falk et al., 1997; Mjaaland et al., 1997; Sandvik et al., 2000; Eliassen et al., 2000; Snow et al., 2001a; Rimstad and Mjaaland 2002). It is well established that apoptosis is an important mechanism of cell death induced by influenza virus both *in-vivo* and *in-vitro* (Lowy 2003). Studies have also shown that caspases are involved in influenza virus replication, because treatment with caspase-3 inhibitors prevented the normal intracellular cleavage of NP protein during virus replication (Zhirnov et al., 1999) and that replication of Influenza A virus was strongly impaired when caspase-3 activity was blocked by a casapse-3 inhibitor or small interfering RNAs (Wurzer et al., 2003). Further studies on ISAV induced apoptosis showed that virus induced apoptosis may not be associated with activation of caspase-3. However, ISAV-induced apoptosis could be prevented by using a broad spectrum caspase inhibitor, Z-VAD-fmk, in SHK-1 cells. This finding suggests that ISAV-induced apoptosis in SHK-1 cells occurs via the caspase activation pathway. Specific binding of ISAV proteins, particularly the protein encoded by segment 7 ORF2, to caspase-8 (Table 3.1) suggest that these proteins may have a role in the apoptosis induced by ISAV.

Since SHK-1 and TO cells are derived from the very target cells of ISAV, the *in-vitro*

observations may be a reflection of *in-vivo* cell death during natural infection. It can be speculated that necrosis of highly susceptible cells such as TO cells *in-vivo* may lead to inflammatory reactions which will result in clinical disease during natural infection. At the same time infection of cell types such as SHK-1 and CHSE-214 cells *in-vivo* may cause no inflammatory reactions, leading to subclinical disease and virus persistence. Therefore, the relevance of this *in-vitro* observation must be investigated *in-vivo.*

Viruses that have the ability to persist within a host need to possess two fundamental characteristics. Firstly, they require the ability to co-exist over time with the host cells they infect, most often being non-cytolytic within such cells. Second, the virus must be able to evolve strategies that allow it to avoid recognition by the host's immune system (Oldstone 1998). In general, virus-specific antibodies play an important role in the control of virus infection in a number of ways. In some instances the presence of specific antibodies can be beneficial to the virus. In this case virus can utilize pre-existing antibodies, to bind to the Fc receptors on phagocytes in order to facilitate infection of their target cells. These viruses usually can replicate in the macrophages or monocytes, and may use them as reservoirs in order to reach other body tissues. Consequently, increased chance to infect target cells results in increased production of viral progeny and often exacerbation of the virus infection or ADE (Sullivan 2001). The common features shared by these viruses include preferential replication in macrophages, ability to establish persistent infection, and antigenic diversity. Previous studies have shown that ISAV exhibits most of these characteristics.

Preliminary virus neutralization studies in our laboratory showed that ISAV is poorly neutralized in macrophage-like fish cell lines by anti-viral antibodies suggesting antibody-mediated growth of ISAV in these cell lines. In this study different methods were used to investigate antibody mediated growth of ISAV in macrophage-like fish cell lines. Virus neutralization using anti-ISAV sera from various sources consistently showed poor neutralization of ISAV in macrophage-like fish cell lines TO and SHK-1 as compared to CHSE-214 cells which used more virus than other two cell lines (Tables 4.1 and 4.2). Complete neutralization of IPNV in TO and CHSE-214 showed that poor neutralization is specific to ISAV in TO and SHK-1 cells. Together these data suggested that poor neutralization of ISAV is due to antibody mediated uptake and replication of the virus in macrophage-like fish cell lines. Further studies using FITC-labeled ISAV and restoration of virus neutralization in TO cells using staphylococcal protein A indicated a possible Fc receptor mediated uptake of antibody-coated ISAV in TO cells. Taken together these data demonstrated that anti-viral antibodies of ISAV can mediate uptake and replication of the virus in macrophage-like fish cell lines possibly through Fc receptors.

Antibody enhanced internalization and growth of influenza A virus NWS (H1N1) has been demonstrated in the cultured murine macrophage-like cell line in the presence of sub neutralizing antiviral IgG (Ochiai et al.,1988). Further studies have shown that HA and NA of Influenza A virus induce antibodies that enhance up take of viruses via Fc receptors in mammalian macrophage-like cell lines (Tamura et al., 1991). There is also a study which reported exacerbation of clinical signs in pigs after challenge with Influenza A virus

following vaccination using a DNA construct expressing an influenza virus M2-nucleocapsid fusion protein (Heinen et al., 2002). Many viruses showing ADE *in-vitro* are also linked to enhanced infection *in-vivo* during natural infection (Sullivan 2001; Tirado and Yoon 2003). Since ISAV in Atlantic salmon seems to target leucocytic cells, endothelial cells or endothelial-associated cells, and macrophages it seems reasonable to speculate that Fc receptor-mediated antibody-dependent enhancement of ISAV infection may occur *in-vivo*, with numerous possible disastrous outcomes. For example, in primary infections it would accelerate the disease process by efficiently and specifically delivering virus to target cells, whereas in vaccinated fish, it would result in enhanced clinical infection and vaccination failure. The presence of non-neutralizing antibodies capable of facilitating virus entry into macrophages or cells expressing Fc receptors may also contribute to persistence by creating a virus reservoir in macrophages.

A major limiting factor in understanding the role of ISAV proteins in ISA pathogenesis is the apparent scarcity of published or commercially available mAbs targeting clinically relevant epitopes on various viral proteins. In this study a panel of ISAV-specific mAbs were characterized (Chapter 5). The mAbs 4A11 and 9A7 were isotyped as IgG_1, mAb 2D3 typed as IgM, and mAb 2B3 was of mixed isotypes IgG_{2b} and IgM (Table 5.1). Based on their reactions in various tests (Table 5.1) mAbs 4A11 and 2D3 were further selected to investigate ISAV epitopes associated with antibody dependent enhancement of infection in macrophage-like fish cell line TO. No enhancement of infection was observed when $TCID_{50}$/100 µl virus was grown in the presence of varying concentration of the mAbs.

Therefore, further studies are necessary to identify ISAV epitopes involved in antibody-enhanced infection of ISAV in macrophage-like fish cell lines.

Failure to react with ISAV proteins in Western blotting suggests that all the selected mAbs may be targeting conformational epitopes. Therefore, more experiments are needed to identify target protein(s) and epitope(s) of these mAbs.

In conclusion, this study demonstrated for the first time that the mechanism of cell death during ISAV infection is cell type specific and that antibodies to ISAV can mediate uptake and replication of ISAV in macrophage-like fish cell lines possibly through Fc receptors. The results obtained in this study provide new and significant data on the interactions between ISAV and fish cell lines derived from the *in-vivo* target cells of the virus, which can contribute to a better understanding of the host-pathogen interactions of ISAV.

6.2. Future directions

This thesis research set a stage for performing future experiments to identify host and viral factors involved in the pathogenesis and persistence of ISAV. This study showed that the mechanism of cell death during ISAV infection is cell type specific since ISAV-induced apoptosis in SHK-1 and CHSE-214 cells and necrosis in TO cells. Experiments are necessary to know whether ISAV-induced apoptosis is due the direct effect of viral proteins or due to the antiviral response of the host cell. ISAV proteins may be individually expressed in fish cell lines in order to study the role of viral proteins in the virus induced apoptosis. Prevention

of apoptosis using a broad spectrum caspase inhibitor, Z-VAD-fmk suggested that caspase activation pathway is involved in the apoptosis induced by ISAV. Further studies to identify the role of various caspases will also help to elucidate the signaling pathways involved in ISAV-induced apoptosis. Studies using primary cultures of target cells of ISAV may provide more information on the *in-vivo* relevance of ISAV-induced apoptosis. Assays such as TUNEL, electron microscopy, or annexin V staining may be carried out in histological sections of target organs of ISAV collected from Atlantic salmon at various stages of virus infection. Such experiments will provide more information on the role of apoptosis in viral pathogenesis *in-vivo*. It is also important to identify specific markers for ISAV-induced apoptosis. For example activation or down regulation of Bcl-2 family genes may be investigated in order to identify markers for ISAV-induced apoptosis. Once identified, these markers can be used to study the role of apoptosis *in-vivo* during natural infection. This study also showed a possible interaction of ISAV proteins with cellular caspases. In order to conclusively prove this interaction experimental systems such as yeast two-hybrid systems may be used (Karimova et al., 2002). Further corroboration can be done using biochemical analyses including GST-pull down assays or co-immunoprecipitation assays (Price et al., 2003). These studies will shed more light on the role of viral proteins in the pathogenesis of ISAV.

Since leucocytes and macrophages are major targets of ISAV in Atlantic salmon future research efforts must be focused on the *in-vivo* relevance of antibody mediated growth of ISAV which has been demonstrated *in-vitro* in this study. Virus neutralization using antisera

from various sources in primary culture of fish macrophages may be useful to correlate *in-vitro* ISAV-macrophage interactions with *in-vivo* phenomena. It is also important to know whether there is an enhancement of infection when fish macrophages are infected with ISAV in the presence of antibodies. This study showed that mammalian as well as fish antibodies can mediated uptake and replication of ISAV in macrophage-like fish cell lines possibly through Fc receptors. An elegant approach will be to use $F(ab')_2$ fragments in virus neutralization assays. $F(ab')_2$ fragments can be obtained by the digestion of immunoglobulins with proteolytic enzymes such as pepsin. Use of $F(ab')_2$ fragments in virus neutralization will confirm Fc receptor mediated uptake of antibody coated ISAV (Halstead and O'Rourke, 1977).

Along with this, antibody enhanced infection of ISAV may be investigated in macrophage-like fish cell lines or primary cultures of fish macrophages, using anti-ISAV sera from various sources, and different multiplicity of infections of the virus. Since mammalian antibodies mediate ISAV uptake in macrophage-like fish cell lines, ISAV-specific mAbs can be used to identify viral proteins which induce production of the harmful antibodies. In the present study, only two mAbs were used to investigate antibody enhanced infection. Characterization of more mAbs specific to various proteins of ISAV is essential to carry out further studies on antibody enhanced infection. Systematic analysis of ISAV proteins by using both neutralizing and non-neutralizing mAbs is also necessary to identify the ISAV epitopes responsible for this phenomenon. The mAbs which were characterized in this study did not react with ISAV proteins in Western blotting, suggesting that mAbs are directed to

conformational epitopes. Use of non-reducing conditions in the Western blot analysis or antigen ELISA using recombinant ISAV proteins might help to identify the viral proteins targeted by these mAbs. Findings from these studies will bear directly on the current attempts to develop successful control strategies against ISAV.

6.3. References

1. BYRNE, P. J., MAC PHEE, D. D., OSTLAND, V. E., JOHNSON, G., FERGUSON, H. W: Haemorrhagic kidney syndrome of Atlantic salmon. J Fish Dis 1998; 21: 81-91

2. DANNEVIG, B. H., FALK, K., NAMORK, E.: Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J Gen Virol 1995; 76: 1353-1359

3. DANNEVIG, B. H., FALK, K., PRESS, C. M.: Propagation of infectious salmon anaemia (ISA) virus in cell culture. Vet Res 1995; 26: 438-442

4. HASTINGS T, OLIVIER, G, CUSACK, R, BRICKNELL, I, NYLUND, A: Infectious salmon anaemia. Bull Euro Ass Fish Pathol 1999; 19: 286-288

5. ELIASSEN, T. M., FROYSTAD, M. K., DANNEVIG, B. H., JANKOWSKA, M., BRECH, A., FALK, K., ROMOREN, K., GJOEN, T.: Initial events in infectious salmon anemia virus infection: evidence for the requirement of a low-pH step. J Virol 2000; 74: 218-227

6. FALK, K., NAMORK, E., RIMSTAD, E., MJAALAND, S., DANNEVIG, B. H.: Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). J Virol 1997; 71: 9016-9023

7. HEINEN, P.P., RIJSEWIK, F.A., de BOER-LUIJITZE, E.A., BIANCHI, A.T.J., Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. J Gen Virol. 2002; 83: 1851-1859

8. HOVBLAND, T., NYLUND, K., WATANABE, ENDRESEN, C: Observation of infectious salmon anemia virus in Atlantic salmon, *Salmo salar* L. J Fish Dis. 1994; 17: 291-296

9. KARIMOVA, G., LADANT, D., ULLMANN, A. Two-hybrid systems and their usage in infection biology. Int. J. Med. Microbiol.2002; 292: 17-25

10. KIBENGE, F. S. B., MUNIR, K., KIBENGE, M. T., JOSEPH, MONEKE. E. Infectious salmon anaemia virus: the causative agent, pathogenesis, and immunity. Ani Health Res Rev. 2004; In press

11. LOWY, R. J.: Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms. Int. Rev. Immunol. 2003; 22: 425-449

12. LYLES, D. S.: Cytopathogenesis and inhibition of host gene expression by RNA viruses. Microbiol. Mol. Biol. Rev. 2000; 64: 709-724

13. LYNGOY. Infectious salmon anaemia in Norway and the Faroe Islands: An industrial approach. In: Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp 97-109.

14. McALLISTER, P.E., DENSMORE, C.L., BARBASH, P.A: Infectious salmon anaemia virus: Injection challenge and waterborne transmission monitored by hematology and polymerase chain reaction assay. 28th Annual Eastern Fish Health Workshop 2003.

15. McGEACHY, S.M., AND MOORE, M.J.: Infectious salmon anaemia in New Brunswick: An historical perspective and update on control and management practices (1997-2002). Washington, DC U. S. Department of Agriculture Animal and Plant Health Inspection Service, U.S.Department of the Interior, U. S. Geological Survey, and U.S.Department of Commerce, National Marine Fisheries Service. In: Miller, Otis; Cipriano, Rocco C., tech.coords.International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA.Tech.Bull.2003; 1902: 145-153

16. MJAALAND, S., RIMSTAD, E., FALK, K., DANNEVIG, B. H.: Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. J Virol 1997; 71: 7681-7686

17. MILLER, O., AND CIPRIANO, R.C. International response to infectious salmon anemia: prevention, control, and eradicationproceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 2003; pp194

18. MURPHY, A.F., GIBBS, E.P.J., HORZINEK, M.C., STUDDERT, M.J. Virus-cell interactions. In Veterinary Virology, 3rd Edition, Academic Press, San Diego, California, USA. 1999; pp 81-92

19. NYLUND, A., HOVLAND, T., HODNELAND, F., NILSEN, LOVIK, P: Mechanisms for transmission of infectious salmon anemia (ISA). Dis Aquat Org 1994; 19: 95-100

20. NYLUND, A., JACOBSE, P: Sea trout (*Salmo trutta*): a carrier of infectious salmon anemia virus (ISAV). J. Fish Biol. 1995; 174-176

21. NYLUND, A: Reservoir species for the infectious salmon anemia virus (ISAV). Proceedings from workshop on Infectious salmon anemia, St Andrews, New Brunswick 29, 1997

22. NYLUND, A., DEVOLD, M., MULLINS, J., PLARRE, H: Herring (*Clupea harengus*): a host for infectious salmon anaemia virus (ISAV). Bulletin of the European Association of Fish Pathologists 2002; 22: 311-318

23. NYLUND, A., KROSSOY, K., WATANABE, K., HOLM, J.A: Target cells for the ISA virus in Atlantic salmon (*Salmo salar* L.). Bulletin of the European Association of Fish Pathologists 1996; 16: 72

24. OCHIAI, H., KUROKAWA, M., HAYASHI, K. & NIWAYAMA, S. Antibody-mediated growth of influenza A NWS virus in macrophage-like cell line P388D1. J Virol; 1988; 62: 20-26

25. OLDSTONE, M.B., Viral persistence: mechanisms and consequences. Current Opinion Microbiol. 1998; 1:436-441

26. PRICE, D., PARK, I., AVRAHAM, H. Methods for the study of protein-protein interactions in cancer cell biology. Methods Mol Biol. 2003;218:255-67

27. RODGER, H. D., RICHARDS, R. H.: Haemorrhagic smolt syndrome: a severe anaemic condition in farmed salmon in Scotland. Vet Rec 1998; 142: 538-541

28. RAYNARD, R. S., MURRAY, A .G., GREGORY, A: Infectious salmon anemia virus in wild fish from Scotland. Dis Aquat Org 2001; 46: 93-100

29. RIMSTAD, E., MJAALAND, S.: Infectious salmon anaemia virus - An orthomyxovirus causing an emerging infection in Atlantic salmon. Apmis 2002; 110: 273-282

30. SANDVIK, T., RIMSTAD, E., MJAALAND, S.: The viral RNA 3'- and 5'-end structure and mRNA transcription of infectious salmon anaemia virus resemble those of influenza viruses. Arch. Virol. 2000; 145: 1659-1669

30. SCAFFIDI, P., MISTELI, T., BIANCHI, M. E.: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002; 418: 191-195

31. SNOW, M., CUNNINGHAM, C. O.: Characterisation of the putative nucleoprotein gene of infectious salmon anaemia virus (ISAV). Virus Res 2001; 74: 111-118

32. SULLIVAN, N. J.: Antibody-mediated enhancement of viral disease. Curr. Top. Microbiol. Immunol. 2001; 260: 145-169

33. TIRADO, S. M., YOON, K. J.: Antibody-dependent enhancement of virus infection and disease. Viral Immunol. 2003; 16: 69-86

34. THORUD, K., DJUPVIK: Infectious salmon anemia in Atlantic salmon (*Salmo salar* L). Bull. Eur. Ass. Fish Pathol 1988; 8: 109-111

35. TAMURA, M., WEBSTER, R. G., ENNIS, F. A.: Antibodies to HA and NA augment uptake of influenza A viruses into cells via Fc receptor entry. Virology 1991; 182: 211-219

36. WURZER, W. J., PLANZ, O., EHRHARDT, C., GINER, M., SILBERZAHN, T., PLESCHKA, S., LUDWIG, S.: Caspase 3 activation is essential for efficient influenza virus propagation. EMBO J. 2003; 22: 2717-2728

37. ZHIRNOV, O. P., KONAKOVA, T. E., GARTEN, W., KLENK, H.: Caspase-dependent N-terminal cleavage of influenza virus nucleocapsid protein in infected cells. J. Virol. 1999; 73: 10158-10163