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Efficacy of rHuIFN-α2b and rFeIFN-ω on Feline Herpesvirus-1 Replication *in vitro*

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ABBREVIATIONS

ACV	Acyclovir
ACV-MP	Acyclovir-Monophosphat
ACV-TP	Acyclovir-Triphosphat
Ara-A	Vidarabine
Ara-C	Cytarabine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
cDNA	Cloned DNA
CPE	Cytopathic effect
CrFK	Crandell Feline Kidney
CTL	Cytotoxic T Lymphocyes
DI water	Deionized water
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DRAF	Double stranded RNA activated factor
dsDNA	Double stranded DNA
dsRNA	double stranded RNA
E-, β-gene	Early gene
EDTA	Ethylen-diamin-tetraacetat
eIF2α	Eukaryotic protein synthesis initiation factor 2 α
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine Serum
Fc receptor	Antibody receptor on macrophages and some NK-cells
FCV	Feline calicivirus
FDA	United States Food and Drug Administration
FeLV	Feline leukemia virus
FHV-1	Feline herpesvirus-1
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FVR	Feline rhinotracheitis virus
g60	Felines glycoprotein, homologous to human gD
GAS	γ-activated site
gB	Glycoprotein B (VP7 and VP8.5)
gC	Glycoprotein C (VP8)
GCV	Ganciclovir
gD	Glycoprotein D (VP17 and VP18)

gE	Glycoprotein E (VP12.3 and VP12.6)
GHV-2	Gallid herpesvirus-2
gI	Glycoprotein I
gp	Glycoprotein
hIFN	Human Interferon
hpi	Hours post infection
HSV-1	Herpes simplex virus type 1
ICP0, - 4, - 8, - 22, - 27, - 47	Infected cell proteins 0, - 4, - 8, - 22, - 27, - 47
IDU	Idoxuridine
IE-, α-gene	Immediate-early gene
IF	Immune fluorescence
IFA	Immunofluorescent antibody assay
IFN	Interferon
IFNAR1 and - 2c	Interferon-α receptor 1 and - 2c
IFNGR1 and - 2	Interferon-γ receptor 1 and - 2
IFNs	Interferons
IL	Interleukin
IM	Intra muscular
IP	Intra peritoneal
IRF-p48/ISGF3	Interferon regulatory factor
ISREs	Interferon-stimulated response elements
Jak 1, Jak2	Januskinase 1, Januskinase 2
kbp	Kilo base pairs
KCS	Keratoconjunctivitis sicca
L-, γ-gene	Late gene
L-valine	Valacyclovir
MHC	Major histocompatibility complex
MLV	Modified-live virus
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTT	3-(4,5-dimethulthiazolyl-2)-2, 5-dipenyltetrazolium bromide
MU	Million units
Mx proteins	Proteins with IFN-inducible GTPases
NK	Natural Killer Cells
OBP	Origin-binding protein
Oct 1	Octamer-binding protein 1
OD	Optical density
oriL, oriS	Large and Small unit of origin of viral DNA

	synthesis
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PFA	Foscarnet
PFU	Plaque Forming Units
PHV-1	Porcines herpesvirus-1
PKR	Protein kinase R
PO	Per os
PRA	Plaque Reduction Assay
Pre-VP22a	viral pre-protein 22a
PTA	Phosphotungstic acid
q12	Every 12 hours
rFeIFN	Recombinant feline interferon
rHuIFN	Recombinant human interferon
RMP	Ribavirin monophosphate
Rpm	Rounds per minute
RTP	Ribavirin triphosphate
SC	subcutane
ss DNA	Single stranded DNA
Stat1, Stat2	Signal transducer and activator of transcription 1, 2
T-25	Tissue culture Flask with 7.5cm^2 surface area
T-75	Tissue culture Flask with 2.5cm^2 surface area
$TCID_{[50]}$	Tissue Culture Infective Dose 50,
TEM	Transmission electron microscopy
TFT	Trifluridine
TK	Thymidine kinase
TMRC	Tetramethylrhodamine isothiocyanate
TNF	Tumor necrosis factor
Tyk 2	Tyrosine kinase
U_L	Unique sequence of the large DNA domain
Us	Unique sequence of the small DNA domain
VAF	Virus-activated factor
vhs	Viral host shut off factor
VI	Virus isolation
VP16	Viral protein 16, alpha-trans inducing factor (α-TIF)
VP19C, -21, - 23, - 24, - 5	Viral protein 19C, - 21, - 23, - 24, - 5
VZV	Varicella-zoster virus
γ1-gene	Partial late gene
γ2-gene	Truly late gene

1. Introduction

Feline herpesvirus-1 (FHV-1)-infection, also known as feline viral rhinotracheitis (FVR) has a world wide distribution in the cat population, with a high incidence in colony cats (>70 %). FHV-1 typically infects and replicates in epithelial tissue of the upper respiratory tract and conjunctiva causing cytopathic lesions. The virus is recognized as one of the most important pathogens of feline upper respiratory tract infections, conjunctivitis and keratitis. Following primary infection, over 80% of cats are unable to eliminate the virus and develop a carrier state, with intermittent episodes of virus shedding. During latency, the virus persists most frequently in the trigeminal ganglion, and the disease can be reactivated following stress and corticosteroid therapy. Successful treatment, particularly of ocular manifestations associated with recrudescent infection such as dendritic ulcers, remains difficult in the cat. Although a number of antiviral drugs are licensed for the use in humans the application for cats is limited. Available antiviral drugs are virostatic, and particularly the systemic application is associated with severe toxic side effects. Lately, use of interferons has been reported for the treatment of viral infections in veterinary medicine.

Interferons are members of the cytokine family that play an important role as mediators of the innate (nonspecific) immune system. Among their multifunctional properties, they have antiviral, antiproliferative and immune regulatory functions. Although there is only minimal data available to support treatment with human alpha interferon (rHuIFN-α) (FULTON AND BURGE, 1985; NASISSE ET AL., 1996A), it has been recommended for the treatment of feline herpesvirus infection (GOULD, 1998; STILES, 2000). A recombinant feline omega interferon (rFeIFN-ω) has become commercially available (SAKURAI ET AL., 1992) and initial studies have assessed the antiviral properties of rFeIFN-ω against canine and feline virus infections (MOCHIZUKI ET AL., 1994, TRUYEN ET AL., 2001). Both these studies reported promising *in vitro* effects of the recently available rFeIFN-ω against FHV-1 infection. MOCHIZUKI and co-investigators (1994) assessed its antiviral activity at 100 U/ml and 1,000 U/ml and TRUYEN and co-investigators (2001) evaluated the anti-FHV-1 effect at 50,000 U/ml.

Objectives of this study were to evaluate and compare the antiviral efficacy across a wider range of concentrations (100 U/ml - 500,000 U/ml) of rFeIFN-ω (Virbagen®-ω) and rHuIFN-α2b, (Intron®-A) on the replication of feline herpesvirus-1 (FHV-1*) in vitro*. This range was chosen in order to repeat previously studied concentrations, to determine whether the drugs have a dose-

dependant response of antiviral activity and which concentration would be the most effective treatment against FHV-1 in CRFK-cells. The species-specificity of type I interferons is well known and therefore, it was suggested that rFeIFN-ω would result in a more profound effect compared to the rHuIFN-α2b due to its homologous nature. In addition to cell and virus culture techniques, a methodology for the plaque reduction assay was established. Furthermore, for the first time antiviral efficacy for interferon was additionally measured by plaque size reductions and is reported in this study. An *in vitro* MTT-Assay was integrated in the experiment to exclude possible cytotoxic effects that could, in principle, contribute to the antiviral effect of both interferons.

2. Literature Review

2.1. Feline Herpesvirus-1

2.1.1. Classification

The herpesvirus family is comprised of five subfamilies: *Alpha-, Beta-, Gamma-herpesvirinae* and an unnamed subfamily. The subfamilies are divided into genera based on DNA sequence and protein homology, but the taxonomy has not yet been fully established (MURPHY ET AL., 1999, ROIZMAN AND PELLETT, 2001).

Feline herpesvirus-1 (FHV-1) has been classified as a member of the subfamily *Alphaherpesvirinae* based on structural, genomic, physiochemical and replicative properties by the International Committee on Taxonomy of Viruses (ICTV, available from: URL:http://www.ncbi.nlm.nih.gov/ICTV). Prototypical members of the alphaherpesvirus subfamily are human herpesvirus-1 (herpes simplex virus, HSV-1; genus *Simplexvirus*) and human varicella-zoster virus (VZV; genus *Varicellovirus*). Besides FHV-1 there are many alphaherpesviruses relevant to veterinary practice, such as the etiologic agents of Marek's disease (GHV-2; unnamed genus) and Pseudorabies (Aujeszky's disease, PHV-1) (ROLLE ET AL., 1993; MURPHY ET AL., 1999).

2.1.2. Characteristics

As most alphaherpesviruses, FHV-1 is composed of the four major structural elements including (a) an electron-opaque core, (b) an icosahedral capsid, (c) a tegument, and (c) an envelope (NASISSE AND WEIGLER, 1997; MURPHY ET AL., 1999). FHV-1's single molecule of linear double stranded DNA (dsDNA) is wrapped around the fibrous spool-like core, which is anchored inside the capsid. The capsid, 1200 Å in diameter, consists of 150 hexameric and 12 pentameric capsomers, a structural characteristic of all herpesviruses. The capsomers are hollow with a channel running along their inner axis. The capsid is surrounded by an amorphous structure called the tegument (ROIZMAN AND FURLONG, 1974; ROIZMAN AND PELLETT, 2001). The outer layer of the virion is formed by a fragile lipoprotein envelope, which carries about 12 viral glycoprotein (gp) spikes (peplomeres) on its surface. These glycoproteins play an important role during the initiation of infection (described in detail 2.1.3.) (ROIZMAN AND KNIPE, 2001).

Within the alphaherpesvirus family, the length (125 – 229 kb), conformation, and base composition (Guanin + Cytosin ratio variation of 32 – 74 %) of the genome varies considerably. The DNA strand of alphaherpesviruses is divided into two singular sequences: (a) the singular large domain (U_L) and (b) the singular small domain (U_S) (ROIZMAN AND KNIPE, 2001). Reiterated DNA sequences occur at both ends of the strand and play a role in producing different genome isomers during viral replication (MURPHY ET AL., 1999). The FHV-1 genome is 126 - 134 kb in length and is composed of a 99 – 104 kb U_L domain and a 27 – 30 kb U_S domain (ROTA ET AL., 1986; GRAIL ET AL., 1991).

The gene products accomplish multiple functions during the life cycle of the virion. Some of these viral proteins interact with host cellular proteins and have become crucial for virus attachment, entry, replication, packaging and budding while other viral proteins block physiological host cell processes and interactions (described in detail in 2.1.3) (ROIZMAN AND KNIPE, 2001). The gene for the FHV-1 thymidine kinase (TK), one of the herpesvirus-encoded enzymes located on the U_L domain, has been studied in detail and has been shown to play an important role during viral replication (NUNBERG ET AL., 1989).

The host range within the alphaherpesvirinae family is highly variable, ranging from a single host species to a broad spectrum of hosts (MURPHY ET AL., 1999). Properties of alphaherpesvirus infection include a short period of replication, rapid spread in cell culture, and a cytopathic effect resulting in destruction of infected cells. All alphaherpesviruses are known to persist in infected host organisms, primarily in sensory ganglia (MURPHY ET AL., 1999). During latency, the alphaherpes genome may be present within the cytoplasm as an extrachromosomal DNA element and may encode a latency-associated gene (MURPHY ET AL., 1999; ROIZMAN AND PELLETT, 2001). During latency FHV-1 is most often found in the trigeminal ganglion (REUBEL ET AL., 1993).

The ability of FHV-1 to establish a lifelong latent infection in cats is similar to that of HSV-1 in humans: following primary infection of epithelial cells HSV is transported retrograde along the sensory neuron axons and into the cell body within a ganglion, where the virus either replicates or establishes latency (PENFOLD ET AL., 1994; ROIZMAN AND KNIPE, 2001). LYCKE and co-investigators (1988) described the transportation of HSV-1 along the cytoskeleton in sensory neurons against the neuronal stream. Upon neuronal damage or activation, the virus is periodically reactivated and becomes infectious again. Most often capsids are transported anterograde back into the epithelial tissue, were the reactivation

may be asymptomatic or may result in a recurrent lesion associated with virus shedding (ROIZMAN AND KNIPE, 2001).

2.1.3. Replication Cycle

The replication of HSV-1 has been studied extensively and most other known alphaherpesviruses follow a similar pattern. The mechanism of replication is categorized as follows: (a) initiation, (b) early gene expression, (c) DNA replication, (d) late gene expression, (e) assembly and (f) release (NASISSE AND WEIGLER, 1997; MURPHY ET AL., 1999; ROIZMAN AND PELLETT, 2001).

Primary infection with FHV-1 occurs predominantly in epithelial cells of nasal mucosa, conjunciva, tonsils and nasal turbinates (CRANDELL AND MAURER, 1958; WOLF, 2000). During initiation, multiple proteins participate in the attachment of the virus to host cells using cellular receptors. For FHV-1 at least 29 proteins have been identified, six of which are glycoproteins in the viral envelope (FERGEAUD ET AL., 1984). The three viral envelope glycoproteins, gB, gC and gD, have been documented to be essential for HSV-1 infection. (a) The primary attachment is initiated by gC and together with gB it binds to glycosaminoglycan residues of the cell surface heparan sulfate. To facilitate cellular entry, the third envelope protein, gD, binds to a cellular co-receptor (ROIZMAN AND PELLETT, 2001). In cats, a gD homolog protein named g60 has been described (MAEDA ET AL., 1992). Three groups of co-receptors have been found to be crucial for HSV-1 entry: (a) members of the TNF receptor family, (b) members of the immunoglobulin receptor superfamily or the recently documented (c) 3-O-sulfated heparan sulfate (SHUKLA ET AL., 1999). Shortly after uncoating of the virion and fusion of the envelope with the plasma membrane, viral Fc receptors (gI and gE) are expressed on the cell‘s surface and the capsid-tegument structure is released into the cytoplasm and transported to the nucleus along the microtubular network (SODEIK ET AL., 1997). At the nucleus, the viral genome enters through the nuclear pores, where the viral DNA rapidly circularizes into the episomal form (MURPHY ET AL., 1999).

Gene expression of the FHV-1 genome is believed to be similar to the HSV-1 in human cells (FENWICK AND ROIZMAN, 1977; FENWICK AND OWEN, 1988). Based on viral gene expression, which occurs at different points in time, three classes of genes are distinguished: (a) immediate-early (IE, α) genes, (b) early (E, β) genes and (c) late (L, γ; partial late γ1- and truly late γ2-) genes. The three classes are

transcribed in sequence by host cellular RNA polymerase II and the viral mRNAs are translated into IE (α-), E (β-) and L (γ-) proteins (MURPHY ET AL., 1999).

Early gene expression of FHV-1 occurs within a few minutes after viral infection. It is induced by two tegument proteins (VP16 and vhs) that are released into the cytoplasm upon entry into the cell. The viral-host-shut-off factor (vhs, exemplified by U_L41) causes nonspecific degradation of cellular mRNA and shuts off the protein biosynthesis in the host cell. To facilitate the initiation process, the second tegument protein VP16 (viral protein 16 or α-TIF, trans inducing factor) activates together with the cellular transcription factor (Oct1), the transcription of the viral immediate early (IE) gene promoter (ROIZMAN AND KNIPE, 2001). By transcription of the IE genes, five α-regulatory proteins (ICPO, ICP4, ICP22, ICP27 and ICP47) are produced, that stimulate subsequent early gene expression. ICP4 and ICP27 initiate and control viral β- or early (E) gene expression and are crucial for viral replication and nucleotide metabolism.

The E gene products (β-proteins) are enzymes that are expressed 4 - 8 hours post infection (hpi) and are mainly responsible for viral DNA synthesis. Seven proteins are involved in viral DNA replication including thymidine kinase (TK) and ribonucleotidreductase, which increase the nucleotide concentration and U_L9 origin-binding protein (OBP/U_L9), single-stranded (ss) DNA-binding protein (ICP8/U_L29), DNA helicase-primase complex (U_L5, U_L8, U_L52), and DNA polymerase. Initially, U_L9 binds to either the ori_L or ori_S element of the viral genome and begins to unwind the DNA to expose a single-stranded loop-like structure. U_L9 and ICP8 recruit the DNA helicase-primase complex, which unwinds the DNA and synthesizes a primer, from which leading-strand synthesis can be accomplished by the viral DNA polymerase holoenzyme. The replication then switches to a rolling circle mode and U_L9 is released from the DNA. Rolling circle replication produces long head-to-tail concatemers of viral DNA, which are cleaved into single genome molecules during packaging (ROIZMANN AND KNIPE, 2001). During the replication and homologous recombination, inverted U_L and U_S-domains may occur and result in viral isomers of the genome (MURPHY ET AL., 1999; ROIZMAN AND KNIPE, 2001).

Following viral DNA-replication the early (β-) proteins promote the expression of the late (y-) genes (including ICP5, gB, and gD. The replication is cis-acting and requires the viral proteins ICP4, ICP27, and ICP8. The L (y-) genes mainly encode viral structural proteins for assembly of progeny viral particles.

The initial stages of viral capsid assembly are partially localized in the cytoplasm, the final steps occur in the nucleus. Once sufficient amounts of nucleocapsid proteins (VP5, VP23 and VP19C), the scaffolding proteins (pre-VP22a and VP21), and the viral protease VP24 are present in the nucleus, the scaffolding proteins join with VP5. VP5 is found in the form of pentamers and hexamers that are linked by VP23 and VP19C triplexes to form the pre-capsid. At this point viral DNA can enter the nucleus (ROIZMAN AND KNIPE, 2001).

The encapsidation of viral concatemers requires a specific terminal sequences. The scaffolding proteins are released and the new capsid matures by budding through the inner nuclear membrane where the virus obtains proteins and the tegument structure. The envelope, which is acquired during the release of progeny virus, is most likely obtained from the outer nuclear membrane, but the mechanism is not yet fully understood. Either the particles move in vesicles or unenveloped through the cytoplasm into the lumen of the endoplasmic reticulum (ER), or in vesicles through the Golgi network where final maturation of the virion glycoproteins occurs. Finally, progeny virus is released through the plasma cell into the extracellular space. In addition to traveling through the intercellular space, progeny virus can spread from cell to cell across cell junctions (ROIZMAN AND KNIPE, 2001). Tissue damage during active FHV-1 infection is due to viral cytopathogenic properties and occurs after progeny virus is released (CRANDELL AND DESPEAUX, 1959; CRANDELL, 1973, CRANDELL ET AL., 1973).

2.2. Feline Herpesvirus Disease

2.2.1. Epidemiology and Pathogenesis

Feline herpesvirus-1 (FHV-1), also known as feline rhinotracheitis virus (FVR), has a world wide distribution in the cat population. The incidence in colony cats is high (> 70 %) compared to the incidence in single-cat households (< 50 %) (MURPHY ET AL., 1999). The virus was first isolated from a cat suffering from feline viral rhinotracheitis (FVR) (CRANDELL AND MAURER, 1958).

The enveloped virion is relatively fragile and very susceptible to heat, drying or disinfectants. It can persist in the environment at room temperature for only about 24 hours in relatively dry conditions (POVEY, 1979; BALDWIN, 1992; HARTMANN, 2000). Low humidity and good disinfection (i.e. household bleach) are preventative measures for the control of the virus (BALDWIN, 1992).

Antigenic similarities within isolated viruses indicate that all strains belong to one serotype (HORIMOTO ET AL., 1992; GASKELL AND DAWSON, 1998) and appear to be restricted to domestic and wild felids (SCHERBA ET AL., 1988; HOFMANN-LEHMANN ET AL., 1996). Together with feline calicivirus (FCV), FHV-1 is recognized as the major cause (> 90 %) of feline upper respiratory infections in cats (GASKELL AND DAWSON, 1998; HARTMANN, 2000). FHV-1 is highly contagious and besides the individual susceptibility, major environmental risk factors for infection include population density (crowding), the intensity of viral exposure and the virulence of the virus strain (GASKELL AND DAWSON, 1998). Kittens from the age of four to six weeks to the age of twelve months are particularly susceptible to FHV-1 infection, as are immune suppressed adult cats (e.g. lactating cats). Cats with underlying disease (e.g. feline leukemia virus; feline immunodeficiency virus) are also very susceptible to FHV-1 (NASISSE, ET AL., 1993).

FHV-1 infection can manifest as an acute or chronic infection after which over 80 % of infected cats are unable to eliminate the virus and become latent carriers (GASKELL AND DAWSON, 1998). FHV-1 has been isolated mainly from trigeminal ganglion in latently infected cats (GASKELL AND POVEY, 1979; GASKELL ET AL., 1985; NASISSE ET AL., 1992). Thus, the virus persists in the cat population and infectious, latent and chronic carriers serve as a source of infection (WOLF, 2000). FHV-1 infection is mainly acquired by mucosal contact with virus containing secretions and excretions from virus shedding cats (GASKELL AND POVEY, 1982; GASKELL AND DAWSON, 1998; WOLF, 2000). The virus enters the host predominantly through oronasal or conjunctival routes and preferentially replicates at temperatures slightly below 37°C. As a result, the infection most often remains localized in superficial epithelial tissue (WOLF, 2000; HARTMANN, 2000) and this may include the corneal epithelium (NASISSE ET AL., 1989A).

Vertical transmission can occur and result in abortion, but more often kittens become infected when their maternal derived antibodies wane (WOLF, 2000). Death and wasting illness during the early perinatal period may also be observed, but are considered a result of debilitation from the disease (BALDWIN, 1992).

2.2.2. Systemic Manifestation

Typical features of FHV-1 infection include upper respiratory tract symptoms, ocular manifestations and reproductive failure (WOLF, 2000). The acute disease manifests more severely in kittens than in adult cats. After an incubation period of about 24 - 48 hours, a sudden onset of paroxysmal sneezing, followed by profuse

serous nasal and ocular discharge, hypersalivation, fever, anorexia and lethargy may be observed (CRANDELL ET AL., 1958, BALDWIN, 1992; MURPHY ET AL., 1999; WOLF, 2000). Titers of acutely infected animals are high and nasal and ocular discharges contain large amounts of virus for up to two weeks post infection (BALDWIN, 1992; WOLF, 2000). The discharge may progress from serous to mucopurulent due to bacterial invasion (WOLF, 2000). Commonly, these symptoms are associated with keratitis, punctuate corneal ulcers and mild conjunctivitis (GASKELL AND DAWSON 1998; WOLF, 2000).

Coughing and dyspnoe frequently seem to be associated with secondary *Bordetella bronchiseptica* infection (WELSH, 1996). Oral ulceration and excessive salivation are rare observations with FHV-1 infections but are commonly associated with FCV (WOLF, 2000).

In most cases, primary FHV-1 infection is self-limiting and symptoms regress within 10 - 14 days (GASKELL AND DAWSON, 1998). Almost every cat develops a chronic or latent carrier state post primary infection and virus shedding may result in recurrence of symptoms or remain asymptomatic for lifetime. In chronic disease, clinical symptoms may follow a waxing and waning cycle and may be less severe than during the acute course of the disease. Recurrence of the disease and episodes of viral shedding are often triggered by stress and can be experimentally induced by corticosteroid treatment (GASKELL AND POVEY, 1973; GASKELL AND POVEY, 1977; WOLF, 2000). Chronic sinusitis and bacterial overgrow may follow severe primary infection and may result in necrosis and distortion of the turbinates (GASKELL AND POVEY, 1977; GASKELL AND DAWSON, 1998). Superficial cutaneous ulceration can occur occasionally, and ulcerative, necrotizing facial dermatitis or stomatitis have been associated with FHV-1 infections in cats (HARGIS AND GINN, 1999; SCOTT ET AL., 2001).

Viremia, generalized systemic infections, and manifestation in abdominal organs and the central nervous system (GASKELL AND POVEY, 1979; WOLF, 2000). In experimentally infected cats characteristic, histopathological findings are inclusion bodies with halos (Cowdry A-type), and inflammatory infiltration of the epithelial tissue (LAVACH ET AL., 1977; GRACIA-KENNEDY, 1998).

2.2.3. Ocular Manifestation

FHV-1 is considered one of the most important ocular pathogens in feline ophthalmology. It is the most frequently encountered cause of conjunctivitis and keratitis in cats (GLAZE AND GELATT, 1991; STILES, 2000; ANDREW, 2001). There is evidence of FHV-1 involvement in the development of symblepharon, proliferative keratoconjunctivitis (eosinophilic keratitis), keratoconjunctivitis sicca (KCS) and corneal sequestration (BISTNER ET AL., 1971; SPIESS, 1985; CHADIEU AND FONCK, 1989; NASISSE ET AL., 1998). The ocular symptoms result from viral cytopathic effects (CPE) in infected epithelial tissue and secondary bacterial invasion. Particularly in adult cats, symptoms may or may not be associated with signs of upper respiratory infection (NASISSE ET AL., 1989A; NASISSE AND WEIGLER, 1997). Ophthalmia neonatorum may be associated with FHV-1 infection (GLAZE AND GELATT, 1991), and the participation of FHV-1 has been suspected in uveitis anterior (MAGGS ET AL., 1999A).

FHV-1 replicates in the conjunctiva and to some degree, in corneal epithelium (NASISSE ET AL., 1989A). Corneal lesions occur in a biphasic pattern in relation to virus replication and release of progeny virus (NASISSE AND WEIGLER, 1997). The pathogenesis of FHV-1-induced stromal keratitis is similar to naturally occurring HSV-1 keratitis in humans. While corneal ulceration is directly related to the cytopathic effect of FHV-1 replication in cells, stromal keratitis may develop as a result of the immune response to the viral antigen inducing stromal damage due to recruited cytotoxic T-Lymphocytes (NASISSE, 1995).

Apart from trigeminal ganglia, FHV-1 has been isolated from corneal tissue of chronic carriers (GASKELL AND POVEY, 1979; NASISSE ET AL., 1992). FHV-1 can be activated by corticosteroid therapy or endogenous and exogenous stress factors (GASKELL AND POVEY, 1977; NASISSE ET AL., 1989A; SLATTER, 2001) resulting in recurrence of virus replication, progressive stromal keratitis, geographic lesions, and/or may result in interstitial edema with deep neovascularization (NASISSE ET AL., 1989A).

Acute primary ocular FHV-1 infection usually consists of severe bilateral conjunctivitis in combination with upper respiratory tract infection in neonatal and adolescent cats (GLAZE AND GELATT, 1991; ANDREW, 2001). The clinical course of the disease may vary considerably and is often associated with other symptoms, such as conjunctival hyperemia, corneal ulceration, blepharospasm, chemosis and profuse serous to purulent ocular discharge (CRANDELL AND MAURER, 1958; BISTNER ET AL., 1971; STILES, 2000). In kittens with severe conjunctivitis, corneal

ulceration and fibrinous exudation a symblepharon, resulting in obliteration of the lacrimal lake and lacrimal puncta can occasionally develop (BISTNER ET AL., 1971; GLAZE AND GELATT, 1991; NASISSE AND WEIGLER, 1997; STILES, 2000). The acute disease in immune competent animals is usually self-limiting and resolves within 10 - 20 days (ANDREW, 2001).

Approximately four days post primary infection diffuse epithelial necrosis, partial loss of the epithelial layer, and a moderate neutrophilic inflammatory response in the substantia propria are typical histopathological findings. Inflammation with marked polymorphonuclear infiltration and intranuclear inclusion bodies are infrequent observations (NASISSE, 1989A AND 1995).

During acute ocular infection, corneal lesions tend to be microdendritic (delicately and diffuse) (GLAZE AND GELATT, 1991); they may occur in a biphasic pattern associated with virus replication and spread within the cornea cells (NASISSE AND WEIGLER, 1997; STILES, 2000). Early corneal ulcers may also be dendritic (small and branching) or geographical (large and irregular), quickly progressing into deep corneal ulcers (MARTIN AND STILES, 1998, STILES, 2000). Microdendritic and dendritic ulcers are considered pathognomonic for FHV-1 infection (GLAZE AND GELATT, 1991; MARTIN AND DIETRICH, 2003). Acute keratitis is usually accompanied by mild conjunctivitis, blepharospasm, and serous to mucopurulent ocular discharge and hyperemia (NASISSE ET AL., 1989A).

FHV-1 keratitis is thought to be induced mainly by reactivation of latent virus. Therefore, it is primarily observed in adult cats and only occasionally in younger cats (GLAZE AND GELATT, 1991; ANDREW, 2001). The ocular infection will commonly result in ulcerative keratitis either in one or both eyes in the absence of respiratory signs. Corneal ulceration in older cats is likely to recur, heal spontaneously or manifest in chronic indolent ulcers resistant to therapy (STILES, 2000). Recrudescent episodes of virus shedding and corneal epithelial ulceration and involvement of corneal stroma may result in fibrosis and scarring and subsequently lead to impaired vision or corneal blindness (NASISSE, 1995). Mild corneal edema and regions of fibrosis may accompany severe and chronic cases and superficial and deep neovascularization might occur (GLAZE AND GELATT, 1991).

The role of FHV-1 in the pathogenesis of eosinophilic keratitis or proliferative keratoconjunctivitis has been reported: by using polymerase chain reaction (PCR), NASISSE and co-investigators (1996B) were able to detect feline herpesvirus-1 DNA in 76 % (45/59) of corneal scrapings from cats with

eosinophilic keratitis. This disease is unique to the cat and horse and manifests as a proliferative, progressive, superficial keratitis (PAULSEN ET AL., 1986). The initial clinical observations include uni- or bilateral, progressive, reddish, pink granulation tissue usually arising from either the temporal or nasal limbus and progressing into a bilateral keratitis covering the whole cornea. In chronic cases, pinkish white deposits of cheesy consistency rich in eosinophilic granulocytes are present (MARTIN AND STILES, 1998). Tear production may be decreased and blepharospasm is present in about 20 % of cases. The conjunctiva and the third eyelid may be severely implicated as well as adjacent corneal tissue (ALLGOEWER ET AL., 2001). Histopathological findings in proliferative keratitis are chronic granulomatous inflammation characterized by infiltration of plasma cells, lymphocytes, numerous eosinophilic granulocytes and occasionally mast cells (GLAZE AND GELATT, 1991; PRASSE AND WINSTON, 1996).

Recently published data also reveals FHV-1 involvement in corneal sequestrum, another unique problem to the cat (NASISSE ET AL., 1998). Corneal sequestrum is also known as necrotizing keratitis, partial mummification, focal degeneration with sequestration, or feline keratitis nigrum and appears on the cornea as a brown-black plaque of unknown etiology (SOURI, 1975; PEIFFER AND GELATT 1976; VAWER, 1981; BLOGG ET AL., 1989; MORGAN, 1994; SLATTER, 2001). The isolated black lesions commonly develop over several months by degeneration of collagen and accumulation of brown pigmentation (KNECHT AND SCHILLER, 1966; GELATT, 1971; SOURI, 1975; VARWER, 1981). Particularly prone to corneal sequestra are Persian, Himalayan, Burmese (GELATT ET AL., 1973), Siamese, and American domestic shorthair cats. This partial breed disposition suggests a genetic basis for the disease (CHAUDIEU AND FONCK, 1989). Corneal sequestrum is occasionally accompanied by entropion, blepharospasm, trichiasis, keratopathy or keratoconjunctivitis sicca (GELATT ET AL., 1973). A causal connection between corneal sequestration and FHV-1 infection is suspected as FHV-1 was detected by polymerase chain reaction (GELATT ET AL., 1973; NASISSE ET AL., 1998). In the histological examination, the sequestrum consists of stromal desiccation and degeneration neighbored by a zone of mononuclear inflammatory cells including lymphocytes, plasma cells, macrophages and, to a lesser degree, neutrophils and giant cells (GELATT ET AL., 1973).

2.2.4. Diagnosis

Primary FHV-1 infection is suspected when characteristic ocular and respiratory signs as described above are observed. However, the only pathognomonic clinical sign during primary infection is the presence of dendritic corneal ulcers (ROBERTS ET AL., 1972). These can be detected by staining with rose bengal (GLAZE AND GELATT, 1991; NASISSE AND WEIGLER, 1997). As chronic and recrudescent FHV-1 infection in adult cats most often occur without respiratory involvement, the clinical diagnosis is more or less presumptive. Dendritic herpes keratitis may be present inconsistently (ROBERTS, 1972).

For the laboratory diagnosis of FHV-1 infection, a number of assays are available. The most frequently used detection methods are the immunofluorescent antibody assay (IFA) (CARLSON AND SCOTT, 1978) and virus isolation (VI) (MAGGS ET AL., 1999B). IFA can either be performed as a direct test (polyclonal fluorescein conjugated antibody) or an indirect test (polyclonal unlabled antibody, that is then reacted with a conjugated antibody). A disadvantage of IFA is its low sensitivity (NASISSE AND WEIGLER, 1997). Samples for IFA should be taken before the eye is stained with fluorescein to avoid false positive fluorescence. This does not occur when tetramethylrhodamine isothiocyanate (TMRC)-conjugates is used instead of fluorescein isothiocyanate (FITC)-conjugates (DA SILVA CURIEAL ET AL., 1991).

Virus isolation (VI), formerly considered the “gold standard”, is indicated when multiple cats show typical symptoms. The sensitivity of this method is influenced by handling techniques and the method is more time-consuming than other diagnostic techniques and should be performed by an experienced laboratory. The virus is usually grown on CRFK-cells and IFA testing can be used to confirm the infectious agent (NASISSE AND WEIGLER, 1997). IFA does not differentiate between field virus and attenuated vaccination strain.

Polymerase chain reaction (PCR) testing is now widely available and has proven to be an extremely sensitive method for the detection of FHV-1. Samples for PCR testing include conjunctival swabs from the inferior cul de sac, corneal scrapings or keratectomy samples. PCR analysis for FHV-1 is based on DNA-amplification of the thymidine kinase gene (REUBEL ET AL., 1993; NASISSE AND WEIGLER, 1997). PCR and nested PCR (repeated amplification cycles) are highly sensitive for the detection of chronic FHV-1 infections (REUBEL ET AL., 1993; NASISSE AND WEIGLER, 1997; STILES ET AL., 1997A, B; BURGESSER ET AL., 1999). TOWNSEND and co-investigators (2004) recently developed a reverse transcriptase-

polymerase chain reaction assay to detect feline herpesvirus latency-associated transcripts in the trigeminal ganglia and corneas of cats that did not show clinical signs. The method is so sensitive that the risk of detecting viral DNA in asymptomatic shedding animals is increased (LUTZ ET AL., 1999).

Cytology for FHV-1 diagnosis may be performed, although it appears to be of limited value. Although intranuclear inclusion bodies appear in high numbers during primary infections, they are rarely observed as they are hard to identify with routinely used stains such as Wright-Giemsa (NASISSE AND WEIGLER, 1997). Instead, the May-Grünwald-Giemsa stain has been recommended for identifying intranuclear inclusions (BISTNER ET AL., 1971).

To date, there is no antigen capture ELISA developed for FHV-1, however, a commercially available ELISA (Herpchek®: Dupont, Wilmington, DE) for human HSV-1 shows 90 % sensitivity and a specificity of 99 % for FHV-1, based on comparison with tissue culture isolation (NASISSE AND WEIGLER, 1997).

Apparently, serum neutralization tests are of limited clinical value as they are difficult to interpret due to the high prevalence of vaccinated cats (NASISSE AND WEIGLER, 1997; GASKELL AND DAWSON, 1998).

2.2.5. Treatment and Prevention

Upper respiratory tract infection in the acute stage of the disease is in most cases self-limiting. In severe cases systemic administration of antibiotic therapy is indicated. The treatment of choice is ampicillin (22 mg/kg q8h) or amoxicillin (22 mg/kg q8 - q12h). Both antibiotics are very effective, have few adverse side effects, and can be given to kittens (HAWKINS, 2003). In cases were *Chlamydia spp., Bordetella bronchiseptica* and *Mycoplasma* are suspected, the application of doxycycline (5 – 10 mg/kg q12h) or chloramphenicol (10 – 15 mg/kg q12h) is recommended (WOLF, 2000; HAWKINS, 2003).

Corneal ulceration requires topical antiviral treatment with Trifluridine (TFT), Idoxuridine (IDU), or adenine arabinoside (Ara-A). The treatment protocol should consist of five to six treatments per day. The treatment can be continued for about two to three weeks at most (HAWKINS, 2003). Trifluridine appears to be the most efficient and is the least toxic antiviral agent tested *in vitro* (NASISSE ET AL., 1989B).

Topical treatment with tetracycline or chloramphenicol ophthalmic ointment is recommended up to four times daily (WOLF, 2000; HAWKINS, 2003). The use of

human interferon has been recommended but IFN dosages seem to vary considerably (GOULD, 1998; STILES, 2000) and there is only minimal data available to support treatment with human alpha interferon (rHuIFN-α) (FULTON AND BURGE, 1985; NASISSE ET AL., 1996A).

Transient conjunctivitis does not require specific treatment, as it is usually self-limiting. Treatment with corticosteroids is contraindicated since there is evidence that topical corticosteroids can worsen the course of the disease (NASISSE ET AL., 1989A; HAWKINS, 2003). Hospitalization and conjunctival grafting of severe cases with deep ulceration may be indicated (BLOGG, 1989). Treatment with oral L-lysine has been reported to be effective against FHV-1 infection at low arginine levels when administered during the acute disease in vitro (MAGGS ET AL., 2000). However, this treatment is problematic in cats, as a deficiency of arginine may result in hyperammonemia, vomitus, ataxia and tetanic convulsions and may lead to coma and death (MORRIS AND ROGERS, 1978; ANDERSON ET AL., 1979; BURNS ET AL., 1981). Only recently, MAGGS and co-investigators (2003) conducted an *in vivo* study in latently infected cats and reported that a once daily oral administration of 400 mg of L-lysine resulted in reduced FHV-1 shedding following changes in housing and husbandry but not following corticosteroid administration. Apparently, this dose caused only a short-term increase in plasma L-lysine concentration but did not alter plasma arginine concentration or induce adverse clinical effects. A recent in vitro study has indicated that bovine lactoferrin may be of some effect against FHV-1 (BEAUMONT ET AL, 2003).

The management of chronic upper respiratory tract infection caused by FHV-1 is problematic and usually requires treatment for many years. Treatment should include topical decongestants to facilitate drainage of secretions, long-term antibiotics (amoxicillin, trimetoprim-sulfadiazine) to treat secondary bacterial invasion and supportive care (WOLF, 2000; HAWKINS, 2003). In severe cases, turbinectomy and frontal sinus ablation might become necessary (HAWKINS, 2003). Supportive treatment should focus on keeping oronasal pathways clear and on frequent removal of ocular and nasal discharge (WOLF, 2000; HAWKINS, 2003). Cats should be kept in a room with a vaporizer to bring relief, especially during episodes of severe congestion (HARTMANN, 2000; HAWKINS, 2003). Mechanical breakdown of adhesions is essential to prevent the formation of symblepharon. Furthermore, topical povidone-iodine (1 – 4 %) may be beneficial but herpetic keratitis may as well be resistant to treatment, and may commonly relapse (SLATTER, 2001).

Cats with eosinophilic keratitis may respond to corticosteroids, but in cases where an association with FHV-1 is suspected, other treatment options should be considered instead (MORGAN ET AL., 1996). MARTIN AND DIETRICH (2003) recommend oral megestrol acetate, 5 mg/day during the first week and 2.5 - 5 mg/week maintenance dosage for the following weeks as treatment of eosinophilic keratitis. In immune-mediated stromal keratitis, topical cyclosporine may improve the clinical signs (MARTIN AND DIETRICH, 2003).

Vaccination cannot prevent the disease, but may decrease the severity of clinical signs. Kittens are usually vaccinated at 8 - 10 weeks, again three to four weeks later and finally after another 12 weeks. Booster vaccinations can be given every one to three years (HAWKINS, 2003). Subcutaneous and intranasal modified-live virus (MLV) vaccines (attenuated) are available, as well as killed (disrupted) virus products (JARRETT AND RAMSEY, 2001). The intranasal MLV vaccine provides more rapid and superior protection, longer lasting immunity and better breakthrough of maternal immunity. It is attenuated to a higher degree than the subcutaneous MLV vaccine and has a higher efficacy, but also carries the risk of post-vaccinal outbreak of infection. It is neither suitable for immune suppressed animals nor for pregnant queens or kittens while maternal immunity persists (GREENE, 1998).

The subcutaneous vaccine produces a slower response and requires at least two follow up vaccinations (GREENE, 1998). Modified live vaccines for subcutaneous application should not be aerolized in front of the cat and spilled vaccine should be washed off, to avoid the risk of virus entry through the oronasal route which may cause infection (HAWKINS, 2003). In some cases, it might be useful to determine the need for vaccination by analyzing the FHV antibody status in serum as it is reported to be prognostic of susceptibility (LAPPIN ET AL., 2002) Queens should be vaccinated before breeding. For pregnant queens and also for cats with FeLV and FIV infection, the subcutaneous inactivated vaccine is reported to be safe, although the protection is much weaker than achieved by MLV products (GREENE, 1998; JARRETT AND RAMSEY, 2001). In multiple cat households where FHV-1 infection is endemic, management, such as separation of kittens and their queens, identification and isolation of carriers, ventilation, < 50 % humidity and immunity strengthening programs can have a considerable influence on the recurrence of FHV-1 infection (JARRETT AND RAMSEY, 2001).

2.3. Antiviral Therapy and FHV-1

A number of antiviral drugs are licensed for the treatment of viral disease in humans. Some of those compounds are useful for the treatment of chronic and recrudescent FHV-1 disease in cats but in order to be effective a frequent administration of the antiviral compound is crucial. As all of the presently available antiviral compounds are virostatic and act only during the replicative phase of the infection, latent FHV-1 remains unaffected (HEIT AND RIVIERE, 1995; GREENE, 1998; CRUMPACKER, 2001).

2.3.1. Nucleoside Analogs

Idoxuridine (IDU, 5-iodo-2'-deoxyuridine), Trifluridine (TFT, 5-trifluoromethyl-2'-deoxyuridine), Cytarabine (Ara-C, cytosine arabinoside, 1-ß-D-arabinofuranosylcyosine), Vidarabine (Ara-A, adenine arabinoside, 9-ß-D-arabinofuranosyladenine), Ribavirin (Virazole), Acyclovir (ACV, 9-[2-hydroxyethoxymethyl]guanine) and Ganciclovir (GCV, 9-[1,3-dihydroxy-2-propoxymethyl]guanine) are nucleoside analogs and act during transcription. After an intracellular phosphorylation, they are incorporated into newly synthesized mammalian and viral DNA strands. This results in the inhibition of viral synthesis but also causes a varying degree of damage to the host (HEIT AND RIVIERE, 1995; GREENE, 1998).

Incorporation of either IDU or its fluorinated compound TFT causes DNA breakage and if transcribed, results in defective proteins. As thymidine analogs, they are active against DNA-viruses (GREENE AND WATSON, 1998). For both compounds, systemic use is precluded as it is associated with significant toxicity such as leucopenia, hepatotoxicity, and gastrointestinal problems (HEIT AND RIVIERE, 1995; CRUMPACKER, 2001). Apparently, TFT is most effective against FHV-1 and has an additional property of better corneal penetration than IDU (NASISSE ET AL., 1989B, 1990; HEIT AND RIVIERE, 1995; GREENE, 1998). IDU is manufactured as solution and as ointment while in some countries TFT is only available as an ointment. Nevertheless, extended topical treatment with either drug may cause irritation or therapy resistant corneal ulcers (GREENE, 1998).

The adenine analog Ara-A and the cytosine analog Ara-C have antiviral efficacy against DNA-viruses. After conversion of Ara-C and Ara-A into their triphosphate form, they act as competitive inhibitors predominantly of the herpesvirus induced DNA polymerase (HEIT AND RIVIERE, 1995). Ara-C and Ara-A are less toxic but

also less effective against FHV-1 compared to IDU and TFT (PAVAN-LANGSTON AND DOHLMAN, 1972; PAVAN-LANGSTON ET AL., 1973; NASISSE ET AL., 1989B). However, their antiviral activity was more effective than that of ACV (NASISSE ET AL., 1989B). Administration of Ara-A may prove problematic as it is poorly soluble and therefore, it has to be given in large fluid volumes over 12 hours (HEIT AND RIVIERE, 1995).

ACV and GCV are synthetic deoxyguanosine analogs with potent anti-herpesvirus activity in humans (HEIT AND RIVIERE, 1995). Initially, cellular enzymes phosphorylate GCV while ACV requires viral thymidine kinase (TK) (2.1.3.) (FYFE ET AL. 1978). Cellular enzymes phosphorylate these compounds subsequently into di- and triphosphates. Herpesvirus infected cells contain higher concentrations of ACV-TP relative to healthy cells, due to the higher affinity of acyclovir for the herpesvirus TK. (HIRSCHBERGER , 1988; HEIT AND RIVIERE, 1995). Acyclo-GTP or Ganciclo-GTP competes with deoxyguanosine triphosphate and their incorporation into the viral DNA-strand results in the inhibition of viral DNA polymerase and termination of elongation (HEIT AND RIVIERE, 1995).

In contrast to ganciclovir, acyclovir shows little toxicity (reversible obstructive nephropathy; local irritation). Side effects of GCV in humans include bone marrow suppression, neutropenia, thrombocytopenia, and central nervous signs (HEIT AND RIVIERE, 1995; GREENE, 1998). ACV can be administered either topically or systemically. In herpesvirus infections in humans, the compound is very effective and associated with minor toxic side effects (COLLUM ET AL., 1980; MCGILL ET AL., 1981). However, the antiviral activity of ACV against FHV-1 is not as effective as it is for other herpesviruses in humans. According to a clinical study conducted by NASISSE and co-investigators (1989B), ACV did not reach significant efficacious plasma concentration in cats, when given systemically. Some FHV-1 strains also showed *in vitro*-resistance to ACV (WEISS, 1989). ACV-Dosages recommended for FHV-1 treatment are 25 mg/kg, q12h, PO (HAWKINS, 2003). The antiviral activity of GCV against most herpesviruses is greater than that of ACV, but it is also more toxic. MAGGS and CLARKE (2004) reported an equivalent anti-FHV-1 in vitro efficacy for GCV and IDU.

Valacyclovir hydrochloride (L-valine, 2 [(2-amino-1,6-dihydro-6-oxo-9H-Purin-9-yl) methoxy] ethyl ester, monohydrochloride) is the hydrochloride salt of the L-valyl ester of acyclovir. It is well absorbed and is rapidly converted to acyclovir, resulting in up to fourfold higher acyclovir serum levels compared to oral acyclovir administration (JACOBSON, 1993). Histological findings indicate nephrotoxicity, and toxic changes in rapidly dividing cell-populations at higher concentrations.

Due to its toxic properties, NASISSE and co-investigators considers the drug unsuitable for use in cats (1997).

The triazole purine analog, Ribavirin inhibits the replication of a broad spectrum of RNA and DNA viruses, including herpesviruses. It mechanism of antiviral action is suggested to occur in numerous ways: Ribavirin 5'monophosphate (RMP) indirectly inhibits the synthesis of guanine nucleotides and ribavirin triphosphate blocks ATP and GTP binding to RNA polymerase (REYES, 2001). Oral and systemic administration may lead to a number of side effects, such as extravascular hemolysis, bone marrow suppression, gastrointestinal-toxicity, and central nervous symptoms (GREENE AND WATSON, 1998; REYES, 2001;). Application by aerosol route reaches only low systemic concentrations. Given orally, the concentration in respiratory secretions reaches higher therapeutic levels (GREENE AND WATSON, 1998).

2.3.2. Other Compounds

Foscarnet (PFA) is a pyrophosphate analog with a wide spectrum of antiviral efficacy against DNA and RNA viruses. PFA is an inhibitor of the nucleic acid polymerase. It acts during transcription but its mechanism is noncompetitive as it binds to the pyrophosphate-binding site instead of the base-binding site. PFA does not require viral TK and therefore is useful against acyclovir-resistant herpesvirus infections (HAWKINS, 2003). MAGGS and CLARKE (2004) investigated the antiviral activity of PFA against FHV-1 and reported it to be relatively ineffective compared to IDU or GCV. Due to its short half-life, PFA needs continuous, intravenous administering in order to be effective. PFA accumulates in bone matrix and possibly penetrates the blood-brain barrier. Most of the compound's excretion occurs with the urine, and it is associated with significant nephrotoxicity (GREENE AND WATSON, 1998).

Specific antiviral acting immunoglobulin may prove beneficial in the treatment of viral infections, especially when applied early during infection. Administration via IM, SC and IP routes are possible, and suitable for puppies and kittens. Immunoglobulin can also be given as plasma IV routes (GREENE AND WATSON, 1998). A beneficial effect of γ-Globuline (Feliserin, Serocat) administration during the acute primary infection has not yet been demonstrated. Nevertheless, local as well as systemic treatment has been suggested (HARTMANN, 2000).

Levamisole and Isoprinosine are immune-modulators, which have been used to treat viral infections. These compounds have shown promise; however,

significant improvement in well-controlled investigations has not yet been reported (GREENE AND WATSON, 1998).

2.4. Interferon

2.4.1. Historical Background

In 1957, Isaacs and Lindenmann infected cells of the chorioallantoic membrane of embryonated hen's eggs with influenza A-virus and described a nonviral protein that was found to render host cells resistant to influenza A-virus as well as to other, superinfecting viruses (ISAACS and LINDENMANN, 1957). Although, NAGANO and coinvestigators (1954) had described the protein earlier, it was named interferon (IFN) by ISAACS and LINDENMANN (1957) due to its viral interference. The discovery of Interferon raised hopes for the availability of an extraordinarily potent antiviral agent, but as the protein is only present in scarce amounts, it took years until it was purified and characterized (KNIGHT, 1976; BERG AND HERON, 1980). With advances in molecular cloning techniques and recombinant DNA technology, it was finally possible to produce reasonable amounts of interferon for further research and therapy purposes. In 1981 several human IFN-α (hIFN-α) subtypes were cloned by WECK and co-investigators (1981). The cloning of human IFN-β (hIFN-β) (DWORKIN-RASTL ET AL., 1982) and murine IFN-γ (m IFN-γ) (GRAY AND GOEDDEL 1983A) followed.

Human interferons were reported to antiviral efficacy against herpes simplex keratitis, either if given alone (NEUMANN-HAEFELIN ET AL., 1975; MCGILL ET AL., 1976; TROUSDALE ET AL., 1987) or in combination with nucleoside analogs (DE KONING ET AL., 1983; SUNDMACHER ET AL., 1984; TAYLOR ET AL., 1991). Topical application of interferon early in the course of HSV-1 infection was reported to reduce the severity and duration of clinical symptoms (HO, 1990) and to shorten the period of virus shedding (SMOLIN ET AL., 1984).

Some natural and recombinant human and feline IFNs showed antiviral properties against selected feline viruses *in vitro.* HuIFN and/or FeIFN showed inhibitory effects against feline leukemia virus (JAMESON AND ESSEX, 1983), rHuIFN-αA, rHuIFN-αA/D and a feline IFN preparation (induced in feline lung cells, challenged with Newcastle Disease Virus) showed some inhibition of FHV-1 and FCV (FULTON AND BURGE, 1985; WEISS, 1989). Recombinant HuIFN-α and FeIFN-ß were also found to have antiviral activity against feline infectious peritonitis (FIP) (WEISS AND TOIVIO-KINNUCAN, 1988; WEISS AND OOSTROM-RAM, 1990). Further in vitro and in vivo studies investigated the effect of rHuIFN-α in

combination with antiviral drugs (CUMMINS ET AL., 1988; WEISS 1989; ZEIDNER ET AL. 1990). For FHV-1, WEISS (1989) was able to demonstrate a strong synergistic antiviral activity of ACV and a rHuIFN-αA/D hybrid *in vitro*. These results resembled those of a previous clinical study of HSV-1-treatment in humans (DE KONING ET AL., 1983).

In 1985, HAUPTMANN AND SWETLY identified a new and distinct class of interferons named HuIFN-ω (HAUPTMANN AND SWETLY, 1985). It is one of the more lately characterized interferons (ADOLF, 1995).

The first recombinant feline interferon (rFeIFN-ω), using a baculovirus-vector, was produced in silkworm larvae (UEDA ET AL., 1993A; SAKURAI ET AL., 1992). *In vivo* studies reported that treatment with rFeIFN-ω had some potential against parvovirus infection in dogs (ISHIWATA ET AL., 1998; MINAGAWA ET AL., 1999; MARTIN ET AL., 2002; DE MARI ET AL., 2003;). Recently, rFeIFN-ω has proven to be of some therapeutic value against FIP when used in combination with glucocorticoids (ISHIDA ET AL., 2004)

To date, there are only few *in vitro* studies available on the anti-FHV-1 activity of rFeIFN-ω (MOCHIZUKI ET AL., 1994; TRUYEN ET AL., 2001). MOCHIZUKI and co-investigators (1994) were able to demonstrate a potential antiviral activity and a dose-dependant response of rFeIFN-ω treatments in cell culture. TRUYEN (2001) assessed the antiviral effect of 50,000 U/ml of rFeIFN-ω against several canine and feline viruses and confirmed a potential antiviral activity of this agent.

There is one *in vivo* study, which assessed the pharmacokinetic properties of rFeIFN-ω and suggested that its pharmacokinetic properties are comparable to those of human interferons (UEDA ET AL., 1993B). The distribution of rFeIFN-ω occurred predominantly in the liver and kidneys; it was catabolized mainly in the kidneys, and was excreted in the urine. A residual accumulation of rFeIFN-ω in the body did not occur. The activity of 2', 5'-oligoadenylate synthetase activity was increased by the interferon for 3 days following intravenous application (UEDA ET AL., 1993B). A Swiss research group evaluated the biological activity of rFeIFN-ω by measuring the Mx-protein-expression following topically and orally rFeIFN-ω treatment in cats. A dose-dependent response for the induction of Mx proteins and a long-lasting effect was found at high concentrations of rFeIFN-ω (BRÄCKLEIN ET AL., 2003). A recent study demonstrated a clinical improvement of ocular FHV-1 manifestations when topically treated with high concentrations of rFeIFN-ω-treated *in vivo* (VERNEUIL, 2004).

2.4.2. Classification and Properties

IFNs are glycoproteins that are produced in homologous cells in response to viral infection (STEWART ET AL., 1980). They are members of the cytokine family and act as important mediators of the initial, innate (nonspecific) and adaptive (specific) immune system (BIRON AND SEN, 2001). Apart from antiviral properties, IFNs have anti-proliferative and immune modulating properties (ABBAS AND LICHTMAN, 2000; BIRON AND SEN, 2001). Initially the IFNs were classified by their cellular origin as leukocyte-IFN (now IFN-α), fibroblast-IFN (now IFN-β) and lymphocyte-IFN (now IFN-γ). With further advances in IFN research, supplementary sources of IFN production were discovered the three major classes were classified as IFN-α, IFN-β and IFN-γ (STEWART ET AL., 1980). Based on their genetic properties, size and origin, two types of IFNs can be distinguished: IFN-α and IFN-β are type I IFNs, while IFN-γ is the only member of the type II IFNs (BIRON AND SEN, 2001). Although the type I and type II IFNs are structurally distinct and use different receptors (see below), their pathways overlap partly, which results in similar biological activities (MOGENSEN ET AL., 1999; ABBAS AND LICHTMAN, 2003). Type I IFNs are heat and acid stabile, and show up to 10-times higher antiviral activity than type II IFNs (FARRER AND SCHREIBER, 1993). They are also the most important mediators of the initial and innate immune system and can be produced by virtually any cell (BARON, 1996; ABBAS AND LICHTMAN, 2003).

The human IFN-α (HuIFN-α) and human IFN-β (HuIFN-β) have been characterized in great detail. A gene family of over 24 genes encodes the genetic information for HuIFN-α; HuIFN-β is encoded by one single gene, located on the same chromosome. Based on the degree of homology, the gene family for HuIFN-α can be further differentiated into: (a) HuIFN-α-1 class with at least 14 proteins and (b) HuIFN-α-2 class with two proteins (BARON, 1996). The proteins consist of about 166 amino acids with molecular weights of about 20 kDa. HuIFN-α and HuIFN-β genes exhibit significant genome and protein homology (ZOON ET AL., 1992).

Recombinant feline IFN-ω (rFeIFN-ω) has been characterized as a type I IFN on the basis of its amino acid identity and classified as an IFN-ω type based on the processing pattern of N-terminal sequence (UEDA ET AL. 1993A) and therefore, remains unrelated to the three major classes of IFNs (IFN-α, -β, -γ) (ADOLF, 1995).

The type II IFN, IFN-γ is very sensitive to low pH (< 5) and high temperatures (> 50 °C). It is produced exclusively by immune cells and can mediate a number of immunoregulatory functions. IFN-γ is an important member of the adaptive immune system, and to a lesser degree than type I IFNs participates in innate immunity (BOEHM ET AL., 1997; BIRON AND SEN, 2001). The human IFN-γ (HuIFN-γ) consists of a homodimer with two identical covalently bound glycosylated polypeptidechains, each with 146 amino acids and molecular weights of 20 - 25 kDa (GRAY AND GOEDDEL 1982, 1983B; NAGATA ET AL., 1987). For the type II IFN, only a single IFN-γ gene has been cloned. There is very little sequence homology within different species, which indicates the narrow host-species specificity of the interferon.

2.4.3. Mechanisms of Antiviral Action

The most powerful stimulus for the transcriptional activation of type I IFNs is viral infection. Specifically, dsRNA, which is expressed during viral replication in infected cells (as described in 2.1.3), induces the release of the repressed type I genes, and subsequently induces type I IFN synthesis (ABBAS AND LICHTMAN, 2003). Other stimuli for IFN-α production in mostly dendritic cells include foreign cells, tumor cells, bacterial cells, and viral envelopes. IFN-β is induced not only by viral, but also by other foreign nucleic acids in most body cells, but mainly secreted by fibroblasts, epithelial cells and macrophages. IFN-γ synthesis is induced by direct stimulation of T-cell receptors by specific antigens or mitogens (MARUCCI ET AL., 1981; BIRON, 1997).

Upon activation, IFN-γ is produced by thymus-derived T-cells and by natural killer (NK) cells (BOCCI ET AL., 1988; BIRON 1997). It can be induced as an initial and adaptive response by foreign antigens to which the T-lymphocytes have been sensitized. For a successful immune response by IFN-γ, cofactors such as CD-28 molecules or tumor necrosis factor-α (TNF-α) are required. Simultaneous stimulation of T-cell receptor and CD28 induces enhanced proliferation and cytokine production of mostly IFN-γ and IL-2, TNF-α. In the regulation of the transcriptional activation, several regulatory factors participate by binding to specific transcription factors, which are activated by phosphorylation (ABBAS AND LICHTMAN, 2003).

Type I and type II IFNs bind to two different kinds of cell surface receptors. Both receptors are members of the type II cytokine receptor family and upon activation share components of their signal transduction pathways (FLINT ET AL., 2000;

ABBAS AND LICHTMAN, 2003). Receptors for both IFN types are expressed on all nucleated cells and receptor numbers vary from 100 - 2000 per cell. Binding of the IFN proteins to their receptors occurs with high affinity (FLINT ET AL., 2000; BIRON AND SEN, 2001). The cell surface receptor for type I IFNs consists of two major polypeptide IFN-type I receptor components (IFNAR1 and IFNAR2c) that bind to the cytokine molecule, and associate with the tyrosine kinase (Tyk2) and Januskinase (Jak1) (LUTFALLA ET AL., 1995; ABBAS AND LICHTMAN, 2003). The type II IFN receptor is also a heterodimer with the major subunits IFN-γ receptor (IFNGR1, and IFNGR2). Successful binding of an IFN-γ molecule results in binding of Jak1 and Jak2 onto the intracellular domains (BIRON AND SEN, 2001). IFN-α, -β and -γ share components of the januskinase/signal transducers and share activators of transcription (JAK/STAT-) signal transduction pathway as their major signaling cascade (DARNELL ET AL., 1994). Binding on either one of the receptors leads to a cascade of tyrosine phosphorylation, of which the following are notable: (a) Tyk2 and Jak1 for IFN-α /-β, and Jak1 and Jak2 for IFN-γ followed by (b) tyrosine phosphorylation of the signal transducers and activators of transcription (STAT1 and STAT2-) proteins for IFN-α /-β and the proteins p91/84 for IFN-γ. The phosphorylated STAT proteins form multimeric complexes that enter the nucleus and associate with another cellular protein, an interferon regulatory factor (IRF-p48/ISGF3), to form defined complexes (KRISHNAN ET AL., 1996). These complexes can bind to specific transcriptional control sequences of IFN-α /-β or IFN-γ inducible genes, called interferon-stimulated response elements (ISREs) for IFN-α /-β-inducible genes and IFN-γ-activated site (GAS) elements for IFN-γ inducible genes (DARNELL, 1994).

There are many interferon-independent pathways in cells that can be activated following expression of viral dsRNA in the infected cell. These pathways are not yet fully understood. However, the dsRNA signals are received by ISREs evading the Jak/STAT factors and a dsRNA-activated factor (DRAF) or virus-activated factor (VAF) binds to the ISRE. As a result, target gene transcription is activated and the responding cells release the produced interferons (FLINT ET AL., 2000).

The most important antiviral action is induced on the site of infection by newly released type I IFNs that successfully bind to surface receptors of cells, which are not yet infected. The type I IFNs do not inactivate the virus directly, but instead interfere by up regulating the expression of antiviral effector-proteins which can lead to innate immunity of the cell (FLINT ET AL., 2000). One of the numerous effector-proteins induced is the inactive proenzyme protein kinase, which requires viral dsRNA or another cellular protein for its activation by autophosphorylation

(CARPICK ET AL., 1997). The dsRNA-activated protein kinase (PKR) can phosphorylate the eukaryotic protein-synthesis-initiation factor (eIF2α) to inhibit protein synthesis. The type I IFNs also induce the 2',5'-oligoadenylate-synthetase, which is activated by viral dsRNA to synthetize 2-5-linked oligoadenylates which in turn activates the RNase L. The concentration of RNase L increases 10 – 1000 fold after IFN treatment and degrades most cellular and viral RNA species. Thereby, it inhibits protein synthesis and the replication of virus in the cell (FLINT ET AL., 2000, BIRON AND SEN, 2001).

Besides these broad spectrum antiviral effects of PKR and RNase-L, the Mx proteins and other guanylate binding proteins have been shown to inhibit specific RNA viruses in humans by interaction with viral nucleocapsids, but their antiviral mechanisms are not yet fully understood (HORISBERGER, 1992; HALLER ET AL., 1998; BIRON AND SEN, 2001). In addition, Type I IFNs induce antiviral responses by enhancing cytolytic activity of NK cells before adaptive immune responses have developed, and by increasing the expression of class I MHC molecules. This facilitates the development of adaptive T-cell responses (TNF-α) (BIRON AND SEN, 2001; ABBAS AND LICHTMAN, 2003). Important IFN-γ inducted antiviral activities include the production of nitric oxide synthase. This has a variety of effects including inhibition of herpesvirus replication (BIRON AND SEN, 2001). Prolonged activity of IFN-induced proteins and all the above-mentioned mechanisms lead to cell death, which inevitably results in a limited spread of progeny virus from cell to cell (FLINT ET AL., 2000).

2.4.4. Availability and Medical Application

In human medicine, interferons have been approved in several countries for the treatment of viral infections and cancer. The currently human-approved IFNs in Europe and the United States are human recombinant IFN-α2a (Roferon®-A) and IFN-α2b (Intron® A). Roferon-A® and Intron®A in humans are licensed for the usage in Hairy Cell Leukemia, Malignant Melanoma, Follicular Lymphoma, Condylomata Acuminata, AIDS-related Kaposi's sarcoma, and Chronic Hepatitis C and B (STUART-HARRIS ET AL., 1992; MANUFACTURERS DRUG INFORMATION). Routinely used application methods for IFNs include intramuscular, subcutaneous and intralesional injections, as well as topical administration (CANTELL and PYHALA, 1976). In addition, IFN-β has received US governmental approval for treatment of relapsing multiple sclerosis (BARON, 1996).

The U.S. Food and Drug Administration (FDA, available from: URL:http://www.fda.org) has not yet approved human IFNs in veterinary medicine. Nonetheless, Roferon®-A has been used in cats for the treatment of non-neoplastic, FeLV-disease. For the treatment of non-neoplastic FeLV-associated disease and ocular lesions, oral administration has been suggested (CUMMINS, 1988). The recommended dosage for rHIFN-α (Intron® A) is 30 IU/ml, q12h, PO. The dosage can be increased in life threatening viral infection to up to 10,000 IU/ml- 20,000 IU/kg in cats (HAWKINS, 2003). rFeIFN-ω (Virbagen® Omega) has recently become available for use in animals in Europe (EMEA/V/C/061/01-02/00/00; EUROPEAN COMMISSION - ENTERPRISE DG - PHARMACEUTICALS: REGULATORY FRAMEWORK AND MARKET AUTHORIZATIONS, NOV. 6, 2001. Available from: URL:http://pharmacos.eudra.org). Virbagen® Omega is approved for veterinary usage in parvovirosis in dogs. The recommended application and dosage is once a day for 3 consecutive days at 5 - 10 MU/ml IV (MANUFACTURERS DRUG INFORMATION).

2.5. Purpose and Design of Study

The use of rHuIFN-α has been recommended for the treatment of feline herpesvirus infection (GOULD, 1998; STILES, 2000). However, at the present time there is only minimal data available to justify this treatment (FULTON AND BURGE, 1985; NASISSE ET AL., 1996A; WEISS, 1989). Before recombinant feline interferon was available, interferons could only be produced in scarce amounts, were of different sources and purity (FULTON AND BURGE, 1985). Recently, the first recombinant feline type I interferon (rFeIFN-ω) was produced in silkworm larvae (SAKURAI ET AL., 1992; UEDA ET AL., 1993A) using a baculovirus vector, and has become commercially available (SAKURAI ET AL., 1992). Initial studies have assessed the antiviral properties of rFeIFN-ω against canine and feline virus infections (MOCHIZUKI ET AL., 1994, TRUYEN ET AL., 2001) and reported promising *in vitro* effects of rFeIFN-ω against FHV-1 infection. MOCHIZUKI and co-investigators (1994) assessed its antiviral activity at 100 U/ml and 1,000 U/ml and TRUYEN and co-investigators (2001) evaluated the anti-FHV-1 effect at 50,000 U/ml.

It was the purpose of this study to evaluate and compare the antiviral efficacy of the recently available feline rFeIFN-ω (Virbagen®-ω) and a human rHuIFN-α (rHuIFN-α2b; Intron®-A) in CRFK-cells against FHV-1 infection *in vitro*. A wider range of concentrations (100 U/ml - 500,000 U/ml) was studied than used in the previous studies of MOCHIZUKI et al. and of TRUYEN et al. mentioned above in

order to repeat the previously tested concentration. Further, as IFN dosages recommended for the use in cats seem to vary considerably (GOULD, 1998; STILES, 2000) this range was chosen to determine whether the drugs have a dose-dependant response of antiviral activity and which concentration would be the most effective treatment dosage. The species-specificity of type I interferons is well known and therefore, rFeIFN-ω would be expected to result in a more profound antiviral effect than the rHuIFN-α2b due to its homologous nature in the feline cell line.

The choice of the cell and virus culture system is always a compromise between the ability of the virus to grow adequately and the drug tolerance of the cells. Previous work has established that CRFK-cells are relatively easy to propagate and are susceptible to FHV-1 (CRANDELL ET AL., 1973; THAM AND STUDDERT, 1985): the virus strain multiplies well in CRFK-cells and produces distinct cytopathic effects (CPEs) and well-defined plaques (CRANDELL AND MAURER, 1958, CRANDELL AND DESPEAUX, 1959). The above suggests that the CRFK-cell/ FHV-1-system is an ideal virus-cell-culture system to evaluate antiviral activity by plaque reduction assay.

The plaque reduction assay (PRA) has been the accepted method of antiviral susceptibility testing ever since Renato Dulbecco introduced it for the use in animal virology in 1952 (FLINT ET AL., 2000). The principle of the method is based on visualizing the antiviral effect microscopically by the suppression of viral plaque formation in the presence of an antiviral agent. Under optimal conditions, i.e. when the virus is distributed evenly and progeny virus is limited to cell-bound spread by a solid overlay, one single plaque results from a single-infectious virion. Thereby the method allows the direct quantification of the antiviral impact of the compound against the infection *in vitro* (SWIERKOSZ AND BIRON, 1995; FLINT ET AL., 2000).

In previous studies, the virus-yield reduction assay has been used for testing the effect of interferon on FHV-1 replication (MOCHIZUKI ET AL., 1994; TRUYEN ET AL., 2001). The virus-yield reduction assay consists of two virus culture episodes. In the first episode, virus-infected cells (high MOI) are exposed to the antiviral compound over a period of 2-3 days. Virus-containing supernatants are subsequently collected and frozen. In the second culture episode, the plaque number is quantified and titers are compared to titers of virus control wells (VOGEL ET AL., 1991). The plaque reduction assay has the advantage that the

procedure is of shorter duration, and an antiviral effect can be directly observed in cell cultures in the presence of the test-compound.

An *in vitro* MTT-Assay was integrated in the experiment as a control to exclude possible cytotoxic effects that could, in principle, contribute to the antiviral effect observed following treatment with either of the two interferons. The MTT-Assay is a rapid method for the detection of a potential cytotoxic effect of a compound on a cell culture (MOSMANN, 1983). To improve the procedure, a standardized plate layout was used. An internal standard on the plate (alive control) and a control with known performance (dead control) were used to validate each individual plate.

3. Materials and Methods

3.1. Materials

All Materials were handled in accordance with the legal requirements for Biosafety Level II Agents in the United States (available from URL:www.cdc.gov/). A list of cell culture media and reagents used in this study is included in the appendix (section 9).

3.1.1. Cell Line

Crandell feline kidney (CRFK)-cells were purchased (1 ml frozen cell suspension of 1.9 x 10^6 cells/ml, in 95 % DMEM and 5 % DMSO), from American Type Culture Collection (ATCC, Manassas, VA, USA. Available through: URL:http://www.atcc.org). The cells were originally derived from feline kidney cortex and propagated as a continuous cell line. CRFK-cells are epithelial-like cells; they adhere to cell culture treated surfaces and form monolayers. The purchased strain was provided free of BVD-virus, mycoplasma, bacteria and fungi.

3.1.2. Virus Strain

Feline herpesvirus-1 (FHV-1) strain C-27, (1 ml freeze-dried virus, reconstituted to the original volume of an approximate titer of $10^{5.95}$ $TCID_{[50]}$/ml) was obtained from ATCC. The virus was originally isolated from throat and conjunctival swabs of a cat with respiratory infection. FHV-1 causes cytopathic effects and intranuclear inclusions in CRFK-cell culture and it induces the formation of mineral crystals in CRFK-cells (CRANDELL ET AL., 1973).

3.1.3. Interferon

Recombinant human Interferon-α2b (rHuIFN-α2b; Intron®-A) was obtained from Schering-Plough (Schering Corporation, Kenilworth, NJ, USA. Available from URL:http://www.schering-plough.com) as a lyophilized preparation (Lot Nr. 2 IFD 303). Each vial contained a powder fraction of10 MU per ml and was diluted in isotonic sodium chloride solution. The original stock solution was stored at 2 - 4°C and a working stock of 5 MU per ml was prepared just before use.

Recombinant feline Interferon-ω (rFeIFN-ω; Virbagen® Omega.) was donated by Virbac SA (Virbac SA, Carros, France. Available from: URL:http//www.virbac.fr). It was received as a lyophilized preparation (Lot Nr. WI22). Each vial contained a powder fraction, which was dissolved in isotonic sodium chloride solution to a final concentration of 10 MU per ml. The original stock solution was stored at 2 - 4°C and a working stock of 5 MU per ml was prepared just before use.

3.2. Cell and Virus Culture Methods

All cell culture work was performed in a Biosafety Cabinet II. During incubation periods, the cells were placed in a controlled, humidified (98 %) incubator (37 °C and 5 % CO_2 /95 % O_2.

3.2.1. Cell Bank

The original CRFK-cell stock contained approximately 1.9 x 10^6 cells per ml. Over several passages, the original cell stock was extended; aliquots were frozen and stored in liquid nitrogen until a representative master cell stock (80 vials, passages 7 and 8) was established and frozen. From the master cell stock, passages were repeatedly cultured as required until sufficient amounts of CRFK-cells were gained to conduct the experiments (passages Nr. 9 – 12). These passages were used as working cell stock throughout the following cell culture procedures and experiments.

3.2.2. Propagation of Cells

CRFK-cells were seeded and cultured in tissue culture flasks (Easy Flask, Nunclon™Δ 75 cm^2 (T-75) and Corning Flask, w/v vented cap 25 cm^2 (T-25)) containing growth medium (D-MEM, high glucose containing 10 % FBS, 100 µg/ml Penicillin, 100 U/ml Streptomycin, 2.5 µg/ml Amphotericin B). The epithelial cells attached to the surface, flattened, grew, split rapidly and formed a confluent sheet of cells (approx. density 8.45 x 10^6 cells per T-75).

The cell cultures were generally split either at or near confluency. To disaggregate cells from each other and detach them from the flask's surface, the growth medium was replaced by a Trypsin-EDTA Solution (0.03 % w/v Trypsin, 0.25 % w/v EDTA). The flasks were placed in the incubator to facilitate the

dispersal of the solution. When the cells became spherical and lifted off the flask surface, twice the volume of growth medium was added to terminate the proteolysis. The cell suspension was collected and centrifuged at 800 × g for 5 minutes to remove the residual dissociation solution. The supernatant was decanted and the cell pellet suspended in growth medium pre-warmed to 37 °C. A sample was taken from this suspension and a cell count and a viability stain were performed (as described in 3.2.4.3.). The split ratio was calculated (either 1:2, 1:3 or 1:5) and the suspension extended to an appropriate volume (see Table 3.1).

Table 3.1. Seeding densities and volume requirements for cell culture propagation

CELL CULTURE VESSEL	SURFACE AREA (cm^2)	SEEDING DENSITY cells/ml	GROWTH MEDIA ml	TRYPSIN-EDTA ml
CORNING FLASK, STERILE; W/V VENTED CAP 25 CM2, VWR CAT.# 29186-010	25	0.8×10^6	5 ml	1.5ml
EASY FLASK, STERILE; NUNCLON™Δ 75 CM2, VWR CAT.# 15708-134	75	2.6×10^6	10 ml	3 ml
BD FALCON, 24-WELL CULTURE PLATES WITH LIDS, STERILE, TISSUE CULTURE TREATED. VWR# 62406-183	2	0.5×10^6	1 ml*	-/-
BD FALCON, 96-WELL CULTURE PLATES WITH LIDS, STERILE TISSUE CULTURE TREATED, VWR# 62406-070	1	0.74×10^6	200 µ*	-/-

*volume per well

3.2.3. Cryopreservation

After Trypsin-EDTA treatment (as described in 3.2.2.) the cells were collected, pooled and centrifuged at 800 × g for 5 minutes. The supernatant was decanted and the pellet suspended in an estimated amount of prepared storage medium (D-MEM, containing 10 % DMSO and 20 % FBS). A cell count was performed (as described in 3.2.4.3.) and the volume extended to a concentration of approximately 2×10^6 cells per ml. One-ml aliquots were dispensed into cryogenic vials, marked and transferred into an incremental cooling device ("Mr. Frosty", Sigma Cat. # C1562) and placed into a -80 °C Freezer for 24 hrs. For long term preservation the vials were transferred to a nitrogen tank where they were stored in the vapor phase (-156 °C) until they were needed and resuscitated. When needed for culture, the cryovials were removed from the nitrogen tank and placed in a water bath at 37 °C. Under rapid agitation of the vial, the frozen cell suspension was thawed within 40 to 60 seconds. The outside

surfaces of the vials were disinfected and aseptically transferred into the hood where the cells were then dispensed at an approximate seeding density of 1 x 10^6 cells per T-25 flask in 5 ml of growth medium and incubated at 37 °C and 5 % CO_2/95 %O_2.

3.2.4. Quality Assurance

3.2.4.1. Microscopic Evaluation

The cultures were examined daily in order to detect contamination (change of color in the medium, bacteria, fungi) and observe the growth, morphology and density of the cells using an inverted microscope (microscopic magnification x 100 – x 400). When contamination of a passage was suspected, cultures were discharged and new passages were resuscitated from the master cell stock and used.

3.2.4.2. Recovery of Frozen Cell Stock

Every one to two weeks after cells had been frozen and stored in liquid nitrogen, one vial of the frozen stock was thawed and the cells were seeded (dilution determined by the concentration of viable cells present at freezing) and observed concerning the recovery time and viability of cells in the culture.

3.2.4.3. Quantification and Viability

Cell viability was assessed by trypan blue exclusion (Gibco, Cat. # 15250-012, trypan working solution 0.03 % w/v) on a routine basis when the cell cultures were split. The concentration of the cell suspension (collected as described in 3.2.2.) was determined by counting cells (initial dilution 1:10) of a known volume and within a defined area (10 squares holding a volume of 1.0 x 10^{-3} ml) using an improved Neubauer hemocytometer (0.1 mm deep) and at a microscope magnification of 100 x. By trypan blue exclusion it was possible to estimate the viability of cells in the dilutions. Stained (dead) and unstained (living, intact) cells were counted separately. If the initial dilution resulted in more than 50 cells per square, further dilutions were prepared to improve counting accuracy.

The concentration of the cell suspension per ml is calculated as follows:

Total number of cells/ml = total number of cell counted in 10 squares x dilution factor x 1000

$$\% \text{ VIABLE CELLS} = \frac{\text{VIABLE CELLS (UNSTAINED)}}{[\text{VIABLE CELLS} + \text{DEAD CELLS (STAINED)}]} \times 100$$

3.2.4.4. Growth Rate and Doubling Time

To determine the doubling time of the cell culture, a cell suspension was collected (as described in 3.2.2.) and diluted in growth medium at two different concentrations (15 ml 1 x 10^5 cells/ml and 15 ml of 5 x 10^4 cells/ml). The cells were seeded into 24-well plates at one ml per well and incubated. After 24 hrs, the medium of two wells of each dilution was removed, the cells trypsinized (described in 3.2.2.) and an aliquot of each well was diluted 1:10 in trypan blue (working solution) and counted using a hemocytometer. This procedure was repeated over six days and samples were taken at 24 hour intervals. The mean cell counts were plotted against the elapsed time and the doubling time was estimated. The number of population doublings was determined as follows:

Number of population doublings = 10 Log (N/N0)

N = Number of cells in growth vessel at end of a period of growth

N0 = Number of cells plated in the growth vessel

3.2.5. Virus Bank

To standardize the virus stock and secure a constant titer the original freeze dried FHV-1 powder fraction was diluted in 5 ml of phosphate buffered saline (PBS) and split into 1 ml aliquots. The vials were placed into the -80 °C freezer and kept as the original virus stock. One aliquot was further diluted (1:100) and divided into 1 ml aliquots and frozen as the master virus stock. To create a working virus stock, four vials of the latter dilution were used to infect subconfluent CRFK- cells prepared in flasks at a low multiplicity of infection (see below). The remaining aliquots were frozen and stored at -80 °C as master virus stock.

3.2.6. Propagation and Storage of Virus

Eight T-25 flasks containing subconfluent monolayers of CRFK-cells were inoculated with 0.5 ml (approx. MOI 0.004) of the virus suspension. The medium was discharged, the cells were washed with PBS, and the virus suspensions were added to each flask. During the inoculation period of 60 min, the flasks were placed on a rocking platform to facilitate a homogenous distribution. Then 5 ml of low serum medium (supplemented D-MEM, high glucose, 2 % FBS) was added to each flask and the flasks were placed into the incubator. For three consecutive days, the cell layers were evaluated microscopically for maximal cytopathic effects (see Figure 3.1(a), 3.1(b) and 3.1(c)). On the third day, the flasks were exposed to three freeze-thaw cycles, to break cell structures open and release virus particles. The contents of each flask were collected, pooled and centrifuged at 1200 × g for 10 minutes. The supernatant was collected and frozen in aliquots and stored at -80 °C. The working virus stock contained a total of 176 aliquots (0.5 ml/vial).

3.2.7. Confirmation of Virus in Culture

3.2.7.1. Cytopathic Effect

Morphological alterations in the CRFK-cell culture were observed using an inverted light microscope (microscopic magnification x 100, x 400) only a few hours (5 - 6 hrs) after inoculation with FHV-1. Cells became spherical, showing cluster appearance and spindle formation in the unstained and unfixed cultures. At 36 hours post infection, cell cultures showed complete virally induced cytopathic effects (CPE +++). By then, most cells had become lysed, few multinucleated giant cells (syncytia) with granulated cytoplasma could be observed and cells had detached from the flask's surface.

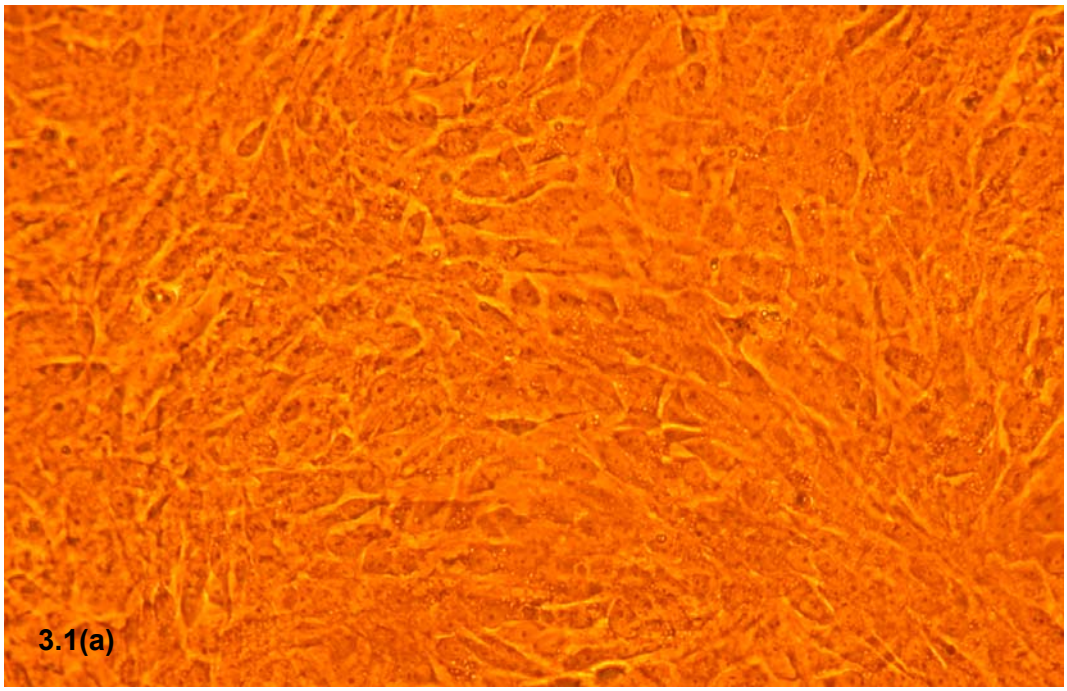

Figure 3.1(a). Confluent monolayer of uninfected CRFK-cells in culture (unstained; microscopic magnification x 400)

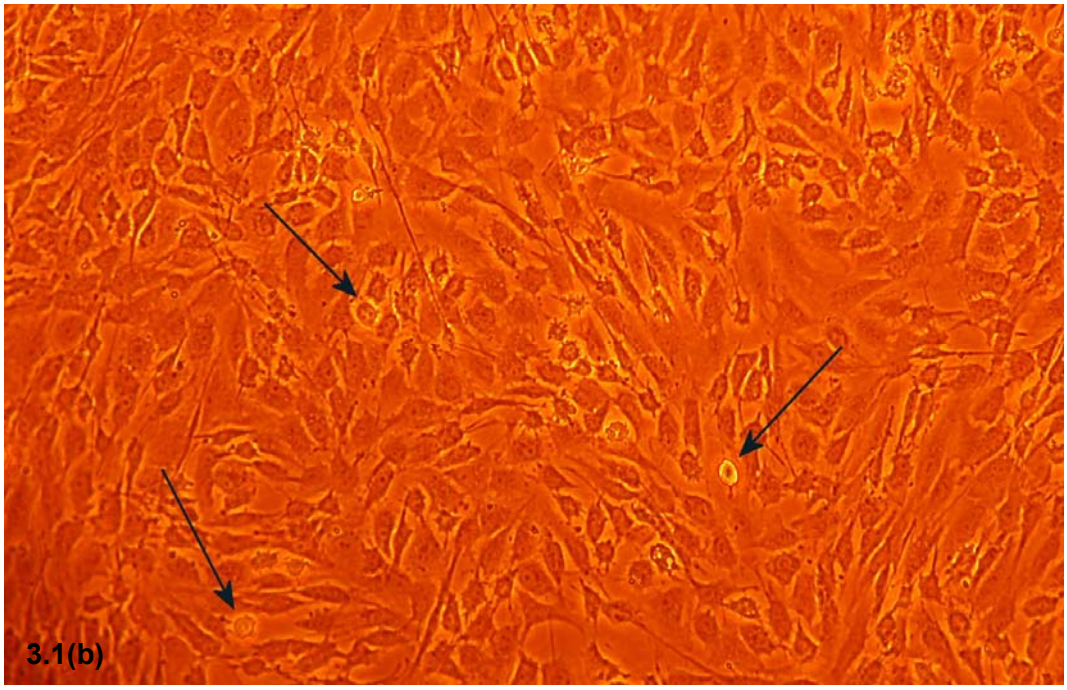

Figure 3.1(b). Confluent monolayer of FHV-1 infected CRFK-cells 6 hours post inoculation in culture. Cells are more distinct and few spherical cells are seen (black arrows) (unstained; microscopic magnification x 400).

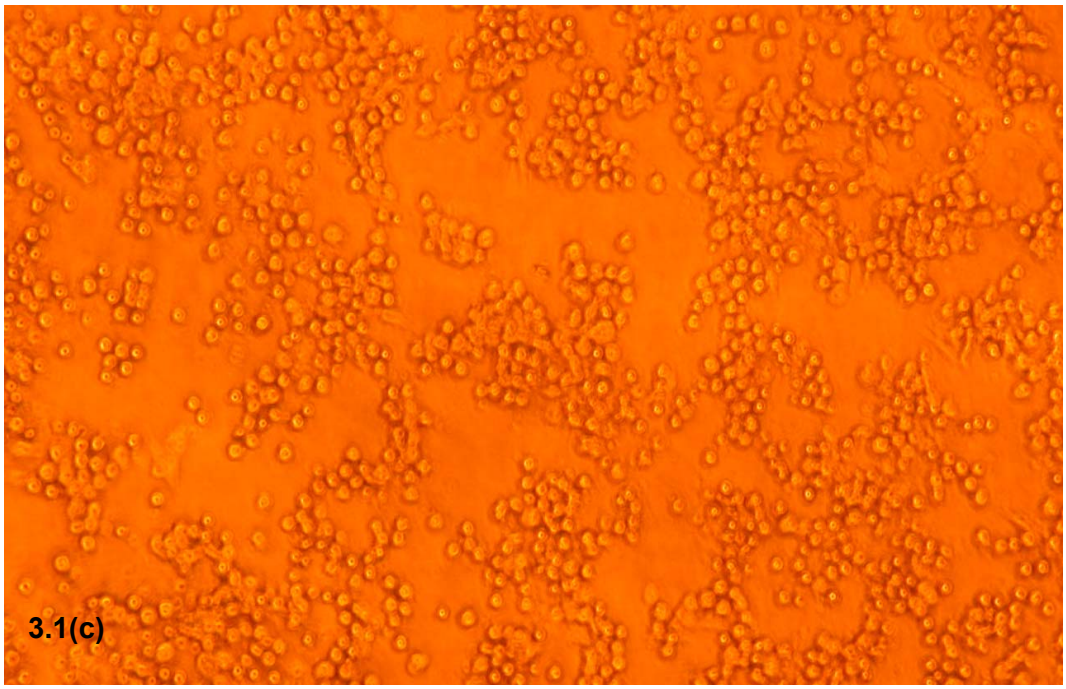

Figure 3.1(c). Cytopathic effects of FHV-1 in CRFK-cells 36 hours post virus inoculation. Cells have become spherical and have detached from the surface of the cell culture flask. Some cells have formed clumps (unstained; microscopic magnification x 400).

3.2.7.2. Transmission Electron Microscopy

A drop of virus containing supernatant (collected as described in 3.2.6.) was placed on a sheet of Parafilm. A formvar-carbon coated electrone microscopy grid was placed on top for 30 min. The grid was removed and excess liquid drained off. Then the grid was touched to a drop of 2 % phosphotungstic acid (PTA) and stained after excess liquid was drained off. The grid was examined with TEM and the virus group was identified by the prominent cubic icosahedral shape of the capsid, the capsomere virus structure and envelope. The examined virus suspension contained numerous negatively stained virus particles. Most of the virions appeared with intact envelopes but to a greater extend virions without envelope could also be observed. The envelopes were distended to various degrees and therefore, the dimensions of the virions varied between 100 - 200 nm in diameter. Most of the nucleocapsids were distinct and intact showing the characteristic cubic icosahedral shape of a herpesvirion while some capsids were disrupted and stain-penetrated. The ultrastructural features of the virion seen by TEM at a microscopic magnification of x 10,000 confirmed the identity of the herpesvirus family.

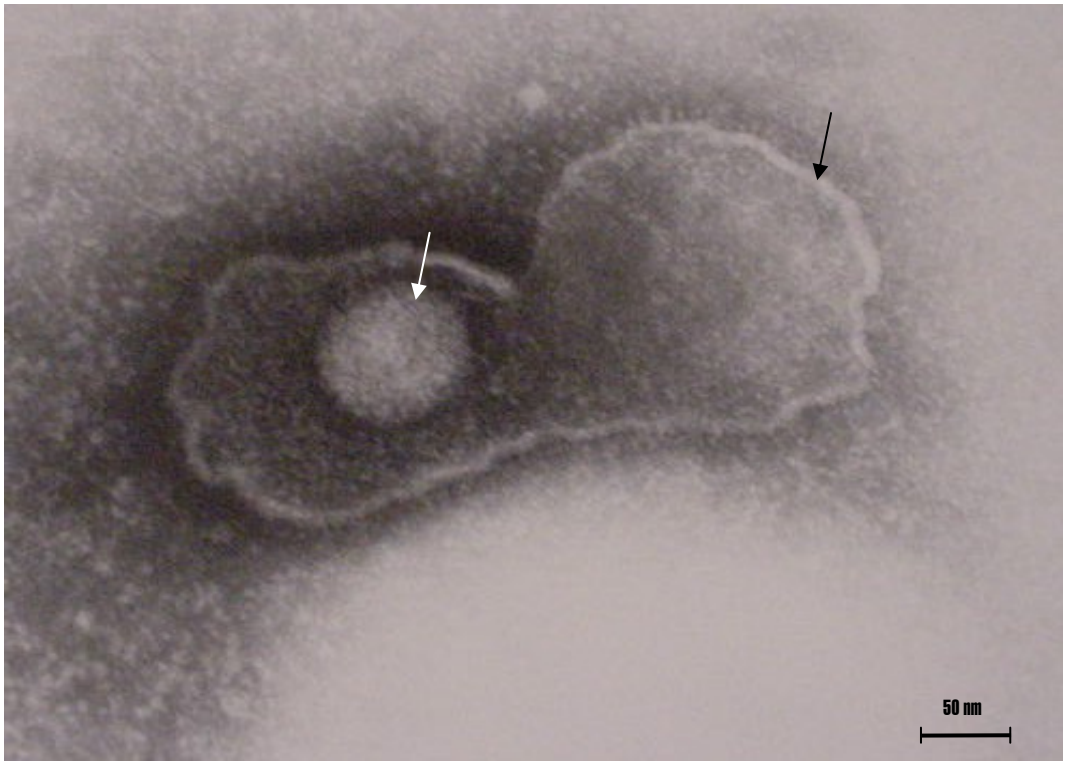

Figure 3.2. The electron micrograph shows a negatively stained FHV-1 virion. Staining and dehydration have distorted the virion. Stain has penetrated the distended envelope (black arrow), The nucleocapsid (white arrow) is intact (microscopic magnification x 10,000).

3.2.7.3. Fluorescent Antibody Staining

A direct immunofluorescent antibody (IFA) staining was performed to identify FHV-1. Therefore, cells at a seeding density of 0.74 x 10^6 cells/ml (200 µl/well) were cultured in 96-well plates for 24 hours. Serial dilutions of virus stock from 10^{-3} through 10^{-10} were prepared. The growth medium was discharged and the cells were washed twice with PBS (200 µl/well). PBS in rows 11 and 12 was replaced by maintenance medium (300 µl/well). In the remaining wells, PBS was replaced by the virus dilutions (inoculation pattern see Figure 3.4). During the inoculation period of one hour, the plates were kept on a rocking platform. Following the inoculation period, the plates were read on an inverted light microscope and dilutions with few CPEs were marked on the plate lid. The plates were washed three times with PBS to discharge virus that had not attached. Maintenance medium was added and the plates were incubated for 48 hrs. Following the incubation period, the suspensions were shaken out of the microtiter plates into a prepared waste vessel and allowed to drain over a sterile paper towel. Remaining media was rinsed out with PBS and the plates were again shaken out over the waste vessel. For the fixation of cells, 80 % acetone (200 µl/well) was added and the plates were put into the freezer (-80 °C) for 20

minutes. The plates were allowed to air dry and the acetone to evaporate. Each well was overlaid with 50 µl of Fluorescein Isothiocyanate (FITC)-labeled antibody (AmericanBioResearchLab, Sevierville, TN, USA, Cat. Nr. 210-41) and incubated at 37 °C for 30 minutes. Prior to reading the wells, the plates were rinsed another three times and shaken out, but residual PBS was allowed to remain in the plates. The microtiter plates were read on the IFA microscope and plaques were identified by the intensive green fluorescent staining of FHV-1 (Figure 3.3).

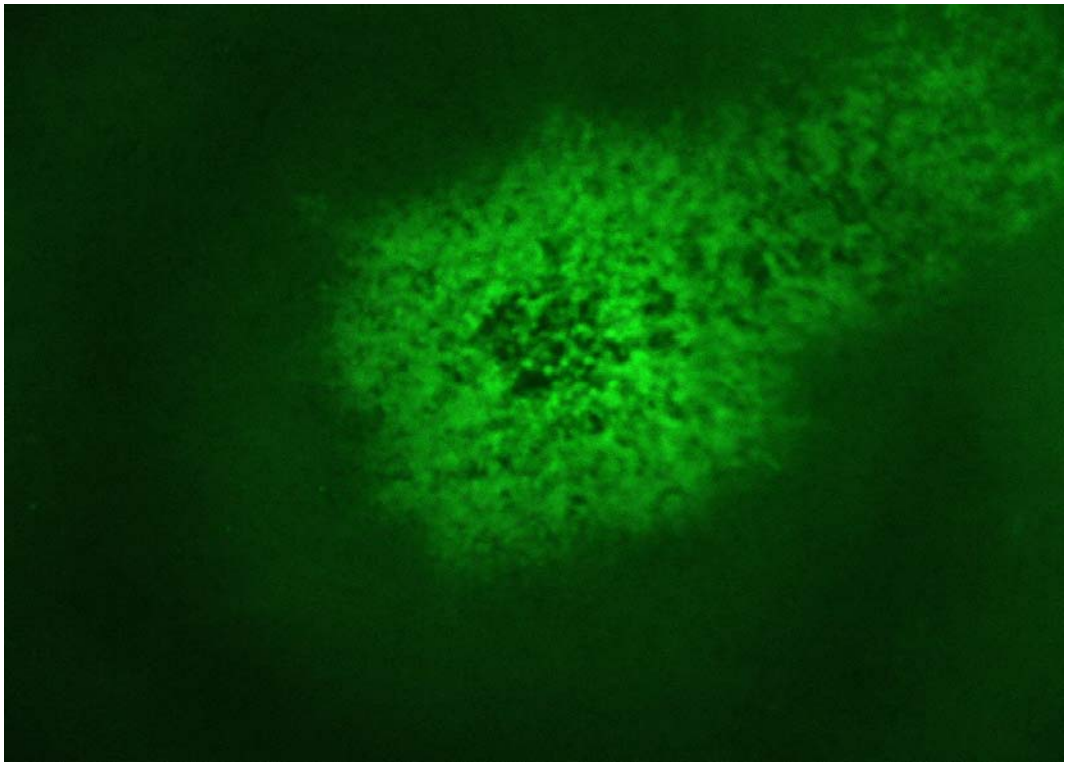

Figure 3.3. Fluorescent microscopic picture of a FITC-stained viral plaque in a CRFK-cell monolayer 4 hrs post FHV-1 inoculation. Viral plaques are distinct and easily identified due to the intensive green fluorescence of the adjoining infected cells (microscopic magnification x 100)

3.2.7.4. Quantal Assay

To evaluate the quantity of the working virus stock, a virus titration was performed in quadruplicate plates (n = 10 per dilution and plate; control n = 112) and repeated several times using five individual samples from the frozen working stock. Therefore CRFK-cells were incubated in 96-well plates for 48 hrs. The virus dilutions were prepared in low serum medium and the assay was performed in 10-fold dilutions from 10^{-1} to 10^{-12} (see Figure 3.4). Following the one hour inoculation period the plates were incubated for 48 hours. The plates were read

using an inverted light microscope (microscopic magnification of x 100 and x 400), CPEs were documented for each well.

The tissue culture infectious dose 50 ($TCID_{[50]}$) was determined for each individual assay using the Reed-Muench Method and an average titer of $10^{8.7}$ $TCID_{[50]}$/ml was determined for the working virus stock (see 9.3 for Reed-Muench Method).

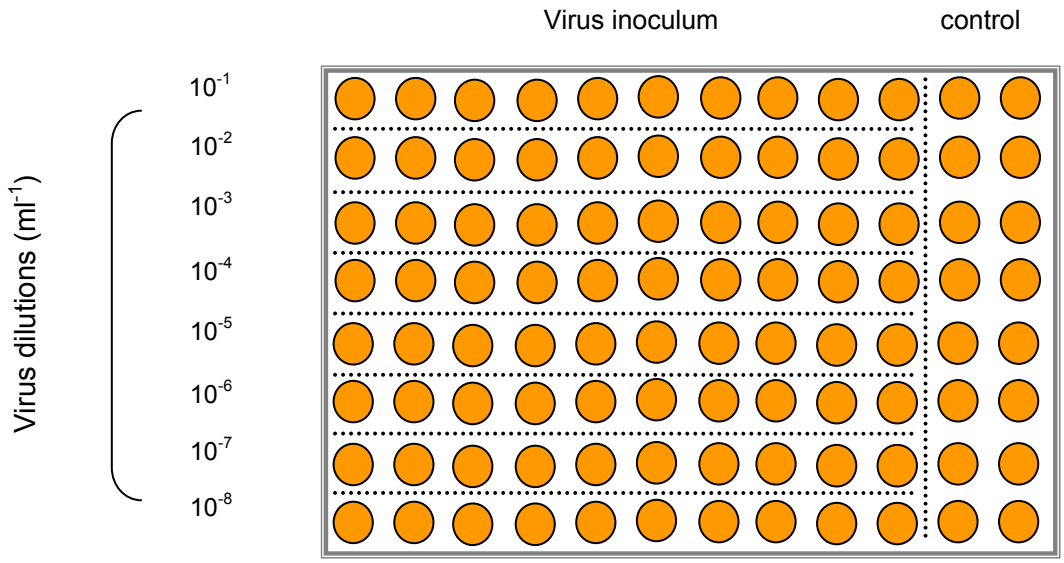

Figure 3.4. Exemplary inoculation pattern for virus titration and IFA-staining. Subsequent dilutions are prepared on additional plates following the same inoculation pattern.

3.3. Experimental Methods

3.3.1. Infectivity Plaque Assay

To standardize the virus suspension (PFU/ml) for the experiments plaque assays were performed in serial dilutions. CRFK-cells were seeded at a density of 0.2 x 10^6 cells ml^{-1} per well into 24-well tissue culture treated plastic plates. After a 24 hr incubation period (at 37 °C and 5 % CO_2/ 95 % O_2), the cells had grown into a 90 % confluent monolayer in each well. The medium was replaced by an inoculum of each virus dilution at 0.1 ml/well. Over the inoculation period of one hour, the plates were placed on a rocking platform to secure a homogenous distribution of virus particles. After removing the virus suspension from the wells, the cells were washed three times with PBS and 1 ml overlay media (1.6 % Seaplaque Agarose 1:1 2X DMEM high glucose, supplemented and with 2 % FBS) was added to each well. Once the overlay media had solidified, the plates were incubated for 72 hrs. For the fixation of cells, 1 ml of a 10 % formalin

suspension was added to each well and left for 4 hrs at room temperature. The formalin was collected and the overlay plugs were gently washed out with deionized (DI) water. To stain the cells, several drops of 0.1 % (w/v) crystal violet were added to each well and rinsed out with DI water after 15 min. The plaques were then counted microscopically (microscopic magnification x 100 and x 400) and expressed as plaque forming units per milliliter (PFUs per ml). The virus challenge dose for subsequent plaque reduction experiments was estimated from the observed relationship between the tested virus dilutions and PFUs per ml as a function of the virus dilution.

3.3.2. Antiviral Plaque Reduction Assay

Several plaque assay protocols were tested and a protocol by LYLE J. BROWN (L.J. BROWN PERSONAL COMMUNICATION, SOUTH DAKOTA 2003) was most suitable for the experiment after modification (i.e. viral input, overlay media formulation, treatments) for the use with CRFK-cells and FHV-1.

Confluent monolayers of CRFK-cells were grown in 24-well cell culture plates. Cells were treated either with rFeIFN-ω (Virbagen® Omega) or rHuIFN-α2b over a set of serial dilutions (100 U/ml, 250 U/ml, 500 U/ml, 1,000 U/ml, 2,500 U/ml, 5,000 U/ml, 10,000 U/ml, 25,000 U/ml, 50,000 U/ml, 100,000 U/ml, 250,000 U/ml and 500,000 U/ml). The interferons were added at three different time points relative to virus exposure in order to maintain the dosages throughout the experiment. The cells were treated six hours before addition of virus, concurrent with addition of virus (30 PFU/ml^{-1}/well), and together with the overlay medium (2X DMEM 1:1 with 1.6 % Agarose). Before each treatment, the cell cultures were washed three times with PBS. Treatments and virus controls were performed in duplicates. Control cell cultures were treated exactly as test cell cultures but received PBS instead of interferons. For each assay, an additional control plate was used to control for possible plate effects. Following incubation for 72 hrs, the cells were fixed with 10 % formalin, overlay plugs were discarded and the remaining cell layers were stained with crystal violet (described above). All plaques per well were counted twice under an inverted microscope (microscopic magnification x 400) and mean plaque counts were calculated from two wells per dilution and assay. Each assay was repeated on six occasions to confirm reproducibility.

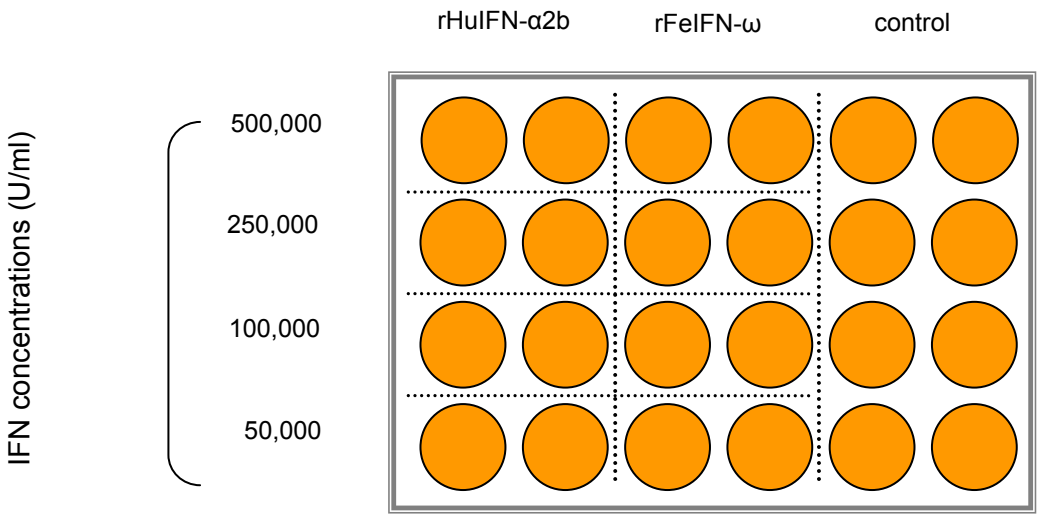

Figure 3.5. Exemplary inoculation pattern used during the plaque reduction assay for IFN-treated test units and virus controls. Subsequent treatments are prepared on additional plates following the same inoculation pattern and plate division.

3.3.3. Plaque Size Reduction

For the evaluation of plaque area reduction, plaques in treated cell cultures (with 100,000 U/ml, n = 20; 250,000 U/ml, n = 40 and 500,000 U/ml, n = 40) and controls (0 U/ml, n = 100) were examined. Plaques were randomly chosen and their diameters (d = 2 x r, with d = diameter and r = radius) were measured using a microscopic measuring reticule. Plaque areas were calculated with the formula $a = r^2 \times \pi$ (with a = plaque area, r = radius), assuming that a circle would best describe their shape. Plaque areas of interferon treated cell cultures compared to controls were analyzed.

3.3.4. Cytotoxic Assay

The MTT-assay is widely used to determine the toxicity of drugs on the cellular level. It measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. It is possible to accurately quantify changes in the rate of cell proliferation because there is a linear relationship between the number of cells and the signal produced in the assay.

The reduction of tetrazolium salt, MTT (3–(4, 5–dimethylthiazolyl–2)–2, 5–diphenyltetrazolium bromide), results in the intracellular accumulation of purple formazan that can be dissolved in an organic solvent and quantified by spectrophotometry. Events that adversely affect cell viability are measured as a decrease in the reduction process of MTT to formazan.

Two assays with 16 replicates per dilution were performed in 96-well cell culture plates, which were seeded with CRFK-cells (density 0.75 x10^6 cells/ml, 200 µl per well). In each of the test wells growth medium was supplemented with either rFeIFN-ω or rHuIFN-α2b (10-fold dilutions ranging from 500 U/ml to 500,000 U/ml). Reference metabolic activity control wells ("alive") received growth medium without rFeIFN-ω and cell-death controls ("dead") were treated with ethanol to obtain 100 % cell death. The plates were incubated at 37 °C for 12 hrs. The medium was aspirated and replaced with 100 µl of medium containing 0.5 mg/ml MTT solution. Following an incubation period of 5 hrs, 90 µl of the medium of each well was removed and replaced with 90 µl of solubilization solution (0.1 N HCL/Isopropanol). The plates were incubated for an additional 5 minutes. To dissolve the formazan crystals, the contents of each well were mixed using a multichannel-pipette. The plates were read at 570 nm and 630 nm. Optical densities (OD) at 630 nm were subtracted from OD at 570 nm and the average corrected ODs were calculated for each dilution and compared with the values of both control groups.

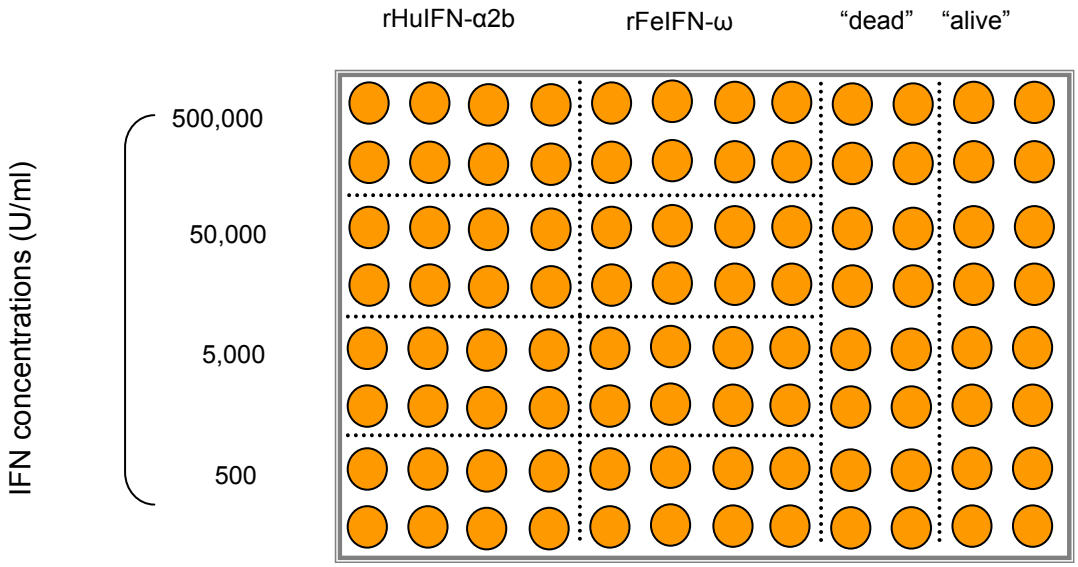

Figure 3.6. Exemplary plate division for MTT-Assay. IFN-treated test units and virus controls are included.

3.4. Statistical Procedures

Regression analysis was used to evaluate the optimal dosage for the virus challenge dosage and trend analysis for plaque reduction and drug dosages. One-way Analysis of variance (ANOVA) was performed using JMP statistical software (JMP,Statistical Software, SAS Institute Inc. Cary, NC, USA).

For drug induced plaque number and plaque size reduction and corrected optical densities obtained in MTT-assay means were compared using a Dunnett's-test with a significance level of $p \leq 0.05$.

4. Experimental Results

4.1. Infectivity Plaque Assay

The virus challenge dose for subsequent plaque reduction experiments was estimated from the observed relationship between the six virus dilutions tested and counts of the plaque forming units (PFUs) per milliliter as a function of the virus dilution. A regression analysis showed that the data was well described by a straight line (r^2 = 0.98). PFU counts increased significantly with increasing virus concentration (decreasing virus dilution) (ANOVA: $F_{1,130}$= 6332, p<0.0001) (see Figure 4.1(a)). Figure 4.1(b) shows the data and the regression line of Figure 4.1(a) plotted with a logarithmic scale for the x-axis. Figure 4.1(b) shows more clearly the lower three dilutions which are compressed together in Figure 4.1(a); the regression line is transformed into a curve as a result of changing to a log scale. The virus dilution of $10^{-6.0}$ (approx. titer of $10^{2,7}$TCID$_{[50]}$/ml) produced an average of 320 PFU/ml. This virus concentration was chosen for the subsequent plaque reduction assay as it produced distinct plaques and plaque counts showed low variability (SEM +/-11.952).

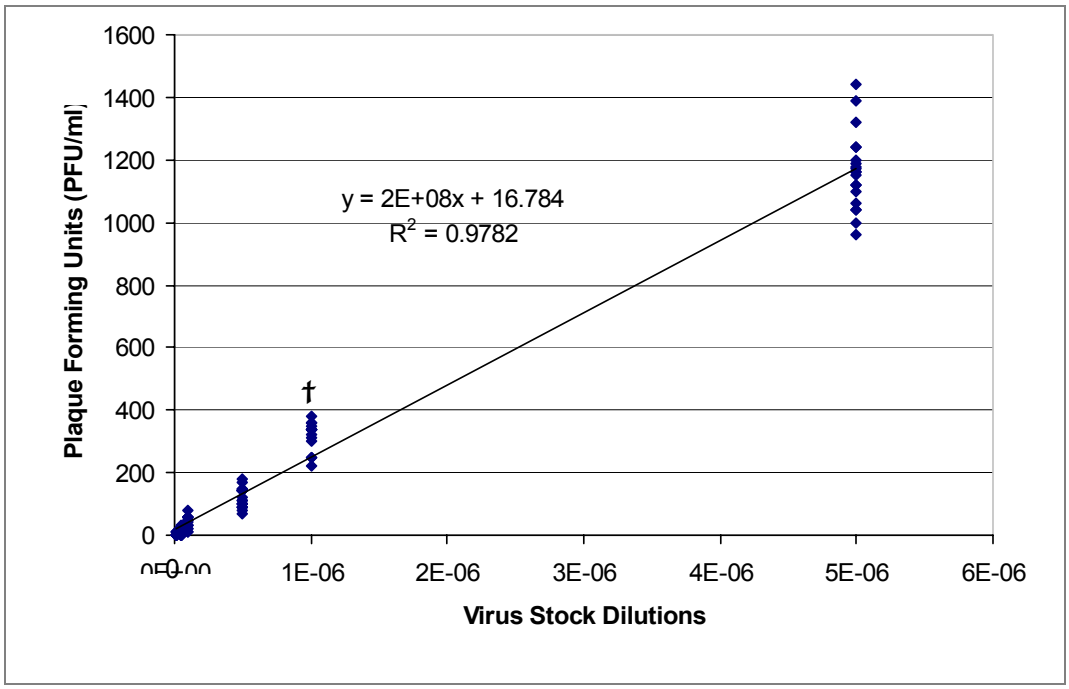

Figure 4.1(a). Relationship between virus dilution (U/ml) and the amount of PFU/ml counted in CRFK-cells across the tested dilutions 72 hours post infection. The equation for the linear regression line is given. Dagger (†) indicates virus challenge dosage producing an average of 320 PFU/ml (approx. $10^{2,7}$ TCID$_{[50]}$/ml). Since the virus dilutions were in approximate log steps, the data is compressed at lower virus concentrations

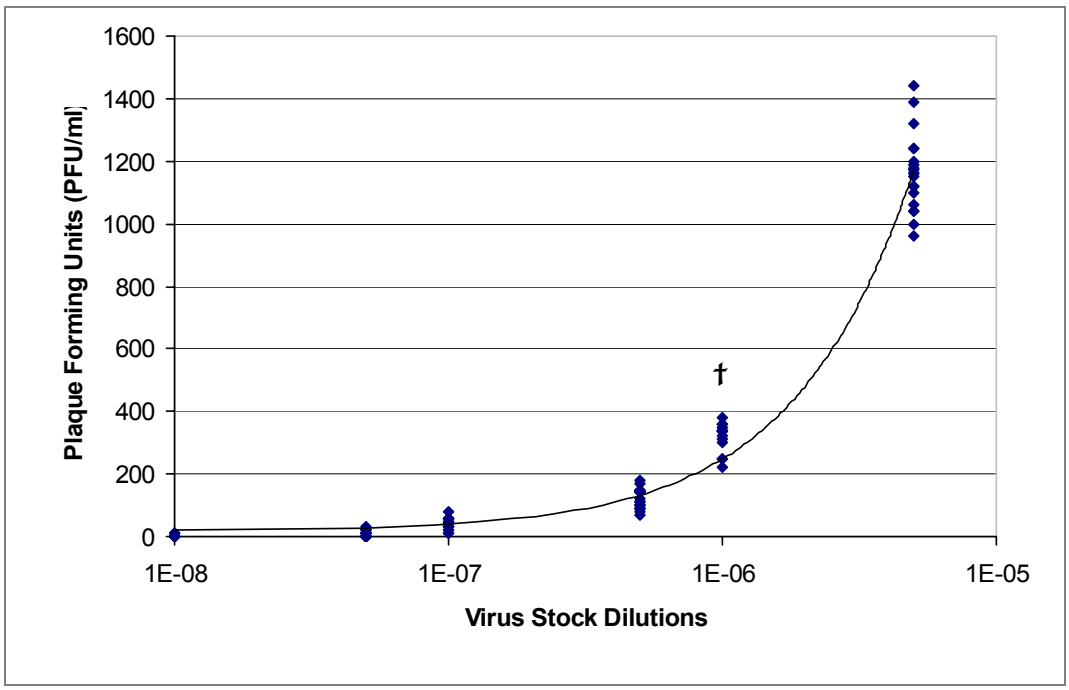

Figure 4.1(b). Same data as in Figure 4.1(a) plotted using a logarithmic scale for the horizontal axis.

4.2. Antiviral Activity of rHuIFN-α and rFeIFN-ω

4.2.1. Plaque Number Reduction

Mean plaque counts for the 12 concentrations per drug and the control condition (untreated CRFK-cells) are shown in Figure 4.2. Dark shaded bars show the counts for rFeIFN-ω (± 1 SEM) and light shaded bars show the counts for rHuIFN-α2b. None of the treatments resulted in a complete suppression of plaques. The effect of the drugs on the plaque formation was assessed statistically for both drugs and each of the twelve dilutions using Dunnett's procedure for testing multiple treatments against a control. This analysis showed that the plaque counts in drug-treated conditions were only significantly different from control counts for the higher concentrations of rFeIFN-ω. The treatments of 100,000 U/ml resulted in a plaque count reduction of 54.7 % ($p < 0.05$) and at 500,000 U/ml a plaque reduction of 59.8 % ($p < 0.05$); these concentrations are marked with asterisks in Figure 4.2. Figure 4.3(a) and Figure 4.3(b) show the mean plaque count reduction (%) relative to the control across all tested IFN treatments including results of the linear regression analysis. Asterisks indicate significant plaque reduction ($p<0.05$) compared to control group. None of the treatments with rHuIFN-α2b resulted in a statistically significant plaque count reduction. However, two of the tested concentrations (5,000 U/ml, $p=0.078$;

100 U/ml, p= 0.099 were found to be close to being statistically significant (p> 0.05 but less than 0.1) but did not reach the requirements to be statistically reliable.

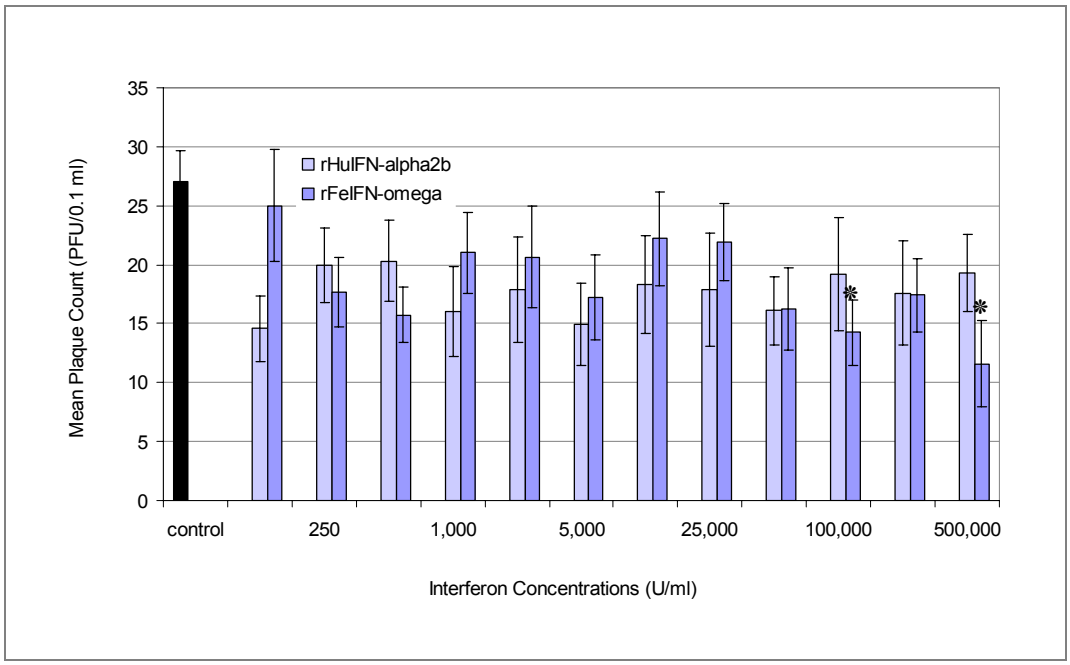

Figure 4.2. Mean plaque counts for all tested IFN-dilution treatments and the untreated control (dark shaded). Dark bars are the mean counts for rFeIFN-ω; light bars represent the mean counts for rHuIFN-α2b. Concentrations are expressed as U/ml. Error bars show ± 1 SEM. Asterisks indicate statistically significant plaque reduction relative to the control condition (p< 0.05)

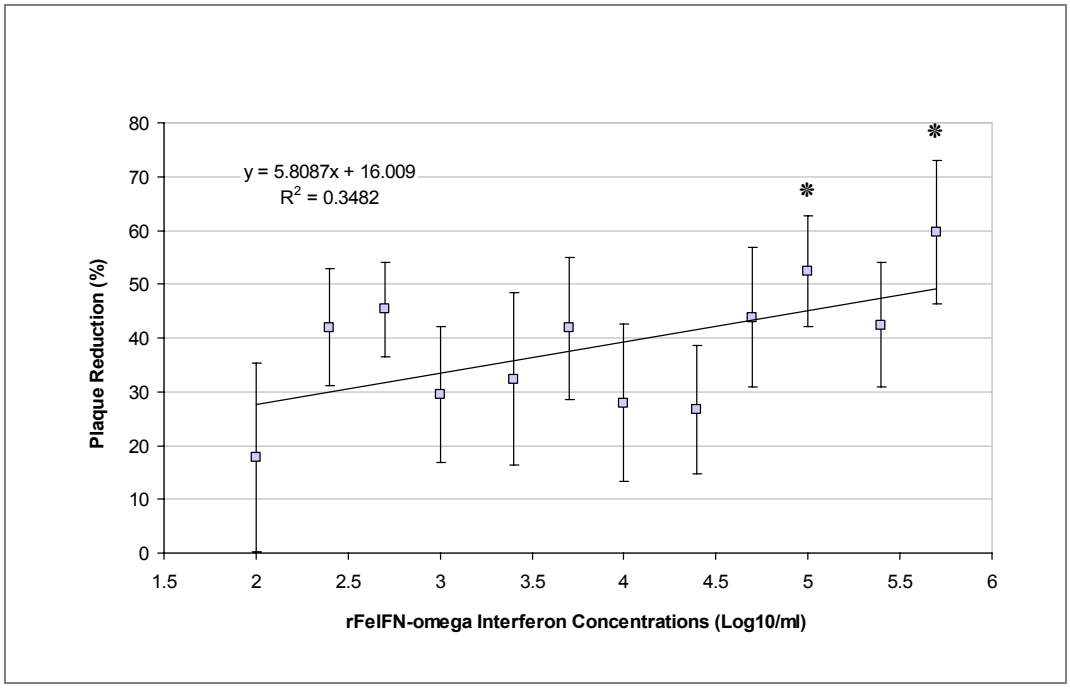

Figure 4.3(a). Mean plaque count reduction (%) relative to the control across all tested IFN-treatments (error bars show ± 1 SEM). Plaque count reduction for the rFeIFN-ω treatments, shown with the results of the linear regression analysis. Asterisks indicate significant plaque reduction (p<0.05) compared to control group

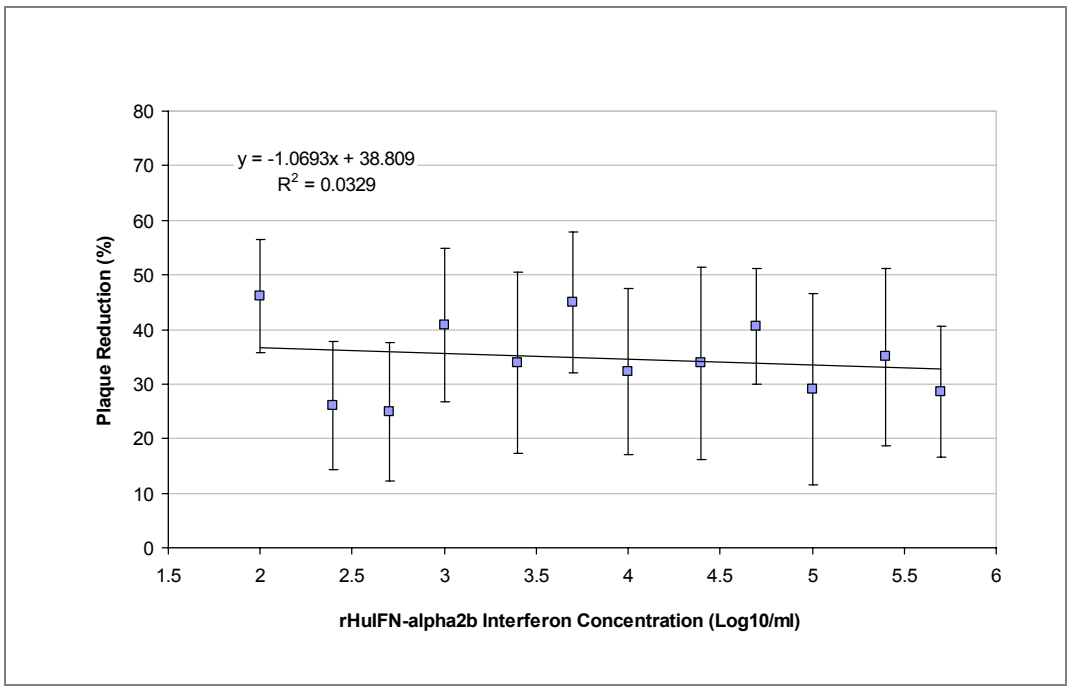

Figure 4.3(b). Mean plaque count reduction (%) relative to the control across all tested rHuIFN-α2b concentrations (Log_{10}/ml), together with the results of the linear regression analysis.

4.2.2. Plaque Size Reduction

Mean plaque areas at each of the drug concentrations were expressed as a percentage of the mean area obtained in the untreated control condition (infected, untreated CRFK-cells). Plaque area reduction for rFeIFN-ω and rHuIFN-α2b at the tested concentrations is shown in Figure 4.4. Plaque area reductions of over 50% were observed in infected CRFK-cell cultures treated with rHuIFN-α2b and for those treated with rFeIFN-ω. Plaque areas at all tested concentrations of rFeIFN-ω and rHuIFN-α2b were found to be significantly reduced relative to the untreated controls ($p<0.05$). The mean percent area reduction for rFeIFN-ω for 100,000 U/ml was 47.5% for 250,000 U/ml 81% and for 500,000 U/ml 70.5%. The mean percent area reduction for rHuIFN-α2b for 100,000 U/ml was 56.4 %, for 250,000U/ml 75.7 % and for 500,000 U/ml 69.8 % (as shown in Figure 4.4).

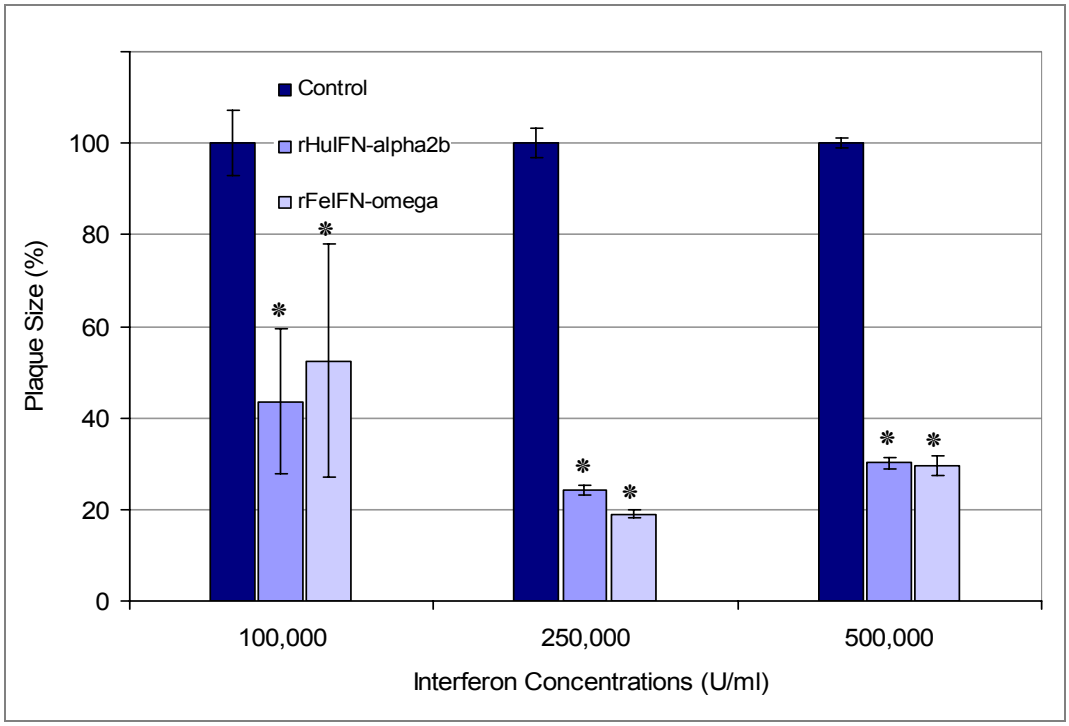

Figure 4.4. Mean percentage of plaque area reduction following either rFeIFN-ω and rHuIFN-α2b treatment with plaque area expressed as percent of the mean control plaque area. Asterisks indicate statistically significant plaque area reduction ($p<0.0001$) compared to infected, untreated cells.

4.2.3. Micrographs

Micrographs were taken in order to document plaque areas of all tested concentrations. Figure 4.5(a) shows the FHV-1 infected untreated CRFK-cell culture with a white-dotted line circumscribing the extension of an average plaque in the control condition. Figure 4.5(b) and 4.5(c) show an average plaque in FHV-1 infected, rHuIFN-α2b (b) and rFeIFN-ω (c) treated CRFK-cell cultures. A white-dotted line circumscribes the plaque areas for better illustration.

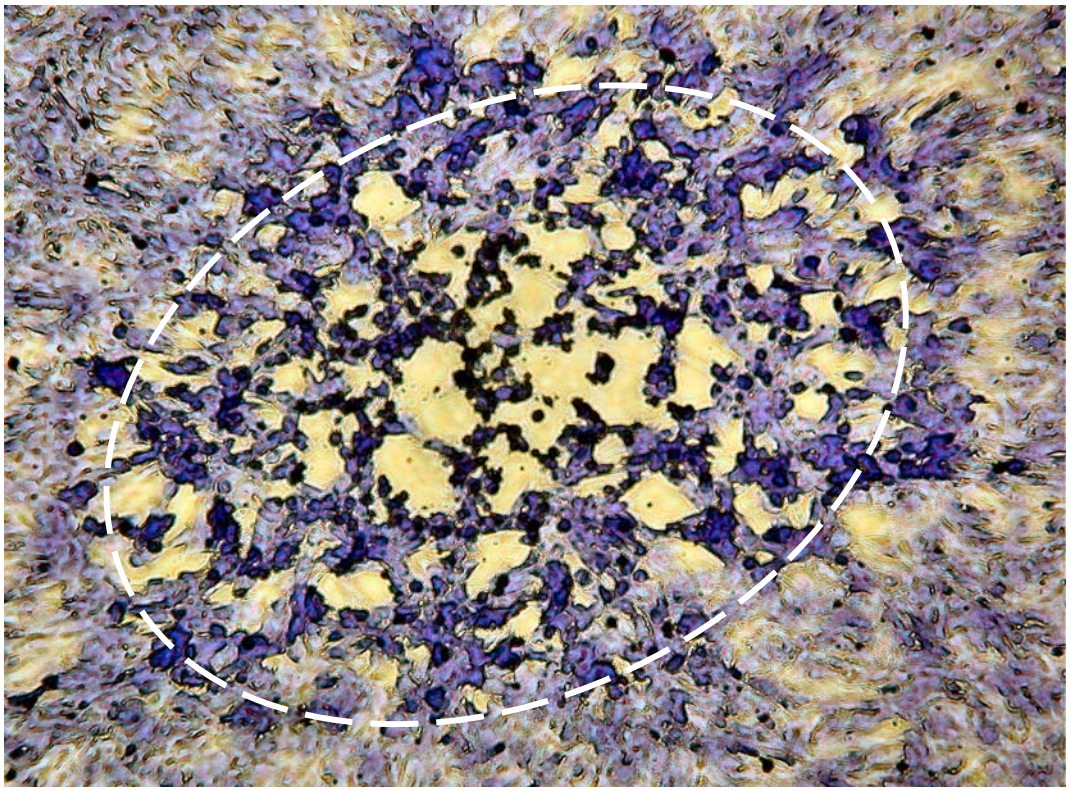

Figure 4.5(a). Micrograph of plaque in infected, untreated CRFK-cell culture. Uninhibited cell-to-cell spread results in significantly larger plaques compared to treated cultures (crystal violet; microscopic magnification x 400)

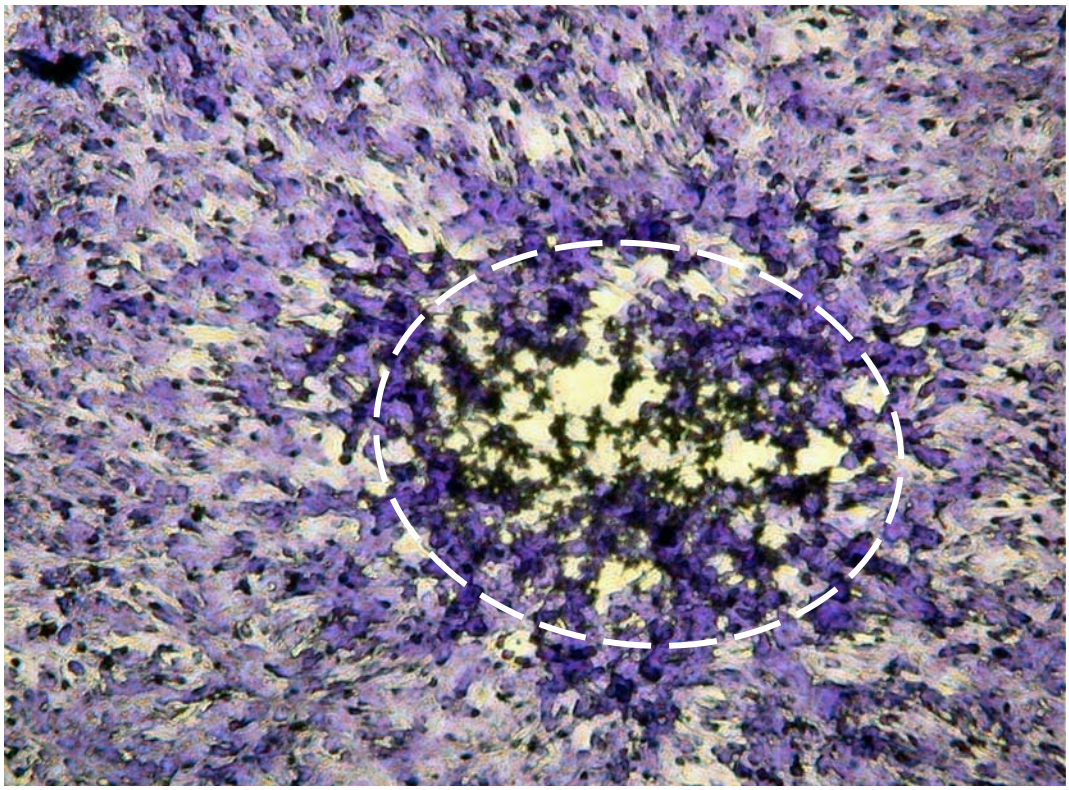

Figure 4.5(b). Micrograph of plaque in infected, rHuIFN-α2b-treated (500,000 U/ml) CRFK-cells in culture. Plaques are significantly smaller compared to control (crystal violet; microscopic magnification x 400)

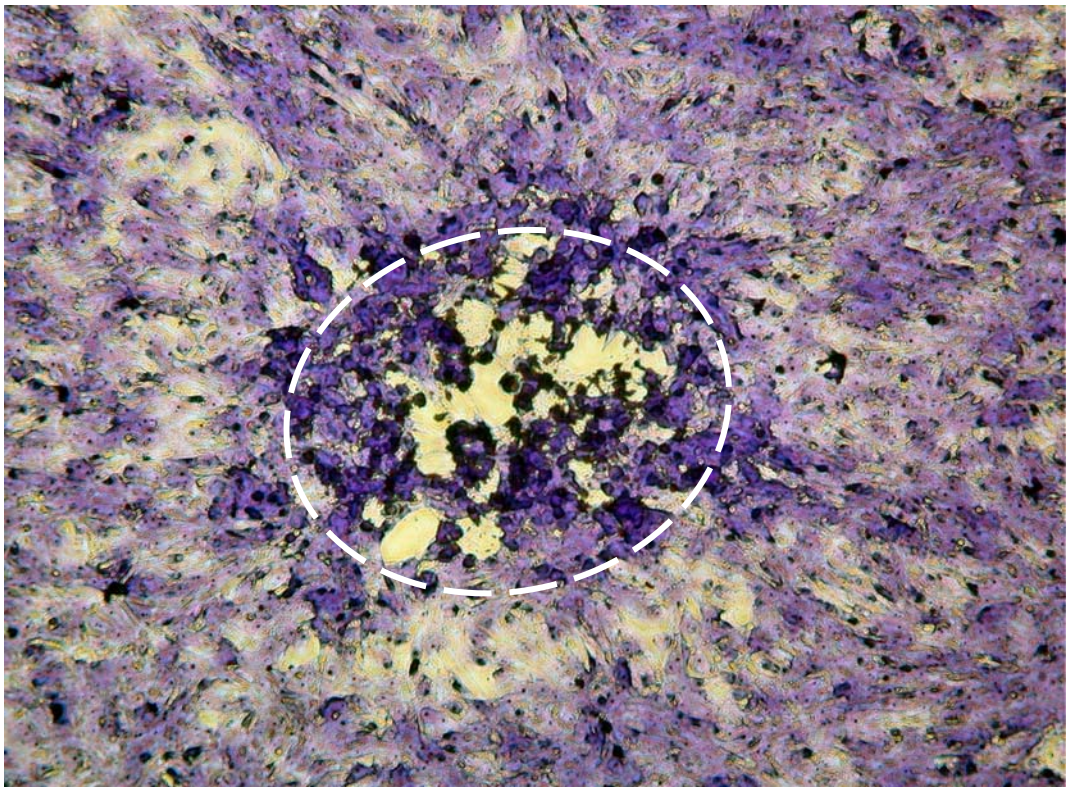

Figure 4.5(c). Micrograph of plaque in infected, rFeIFN-ω-treated (500,000 U/ml) CRFK-cell culture. Plaques are significantly smaller than in untreated cells. (crystal violet; microscopic magnification x 400).

4.3. Cytotoxic MTT-Assay

Neither rFeIFN-ω nor rHuIFN-α2b treatment caused significant cellular toxicity at the tested concentrations (500U/ml; 5,000U/ml; 50,000U/ml; 500,000U/ml). Optical density of rFeIFN-ω or rHuIFN-α2b treated cells were not significantly different from the cells in the medium alone ("Alive"-control) but were significantly different from death control cells ($p<0.05$, Figure 4.6.).

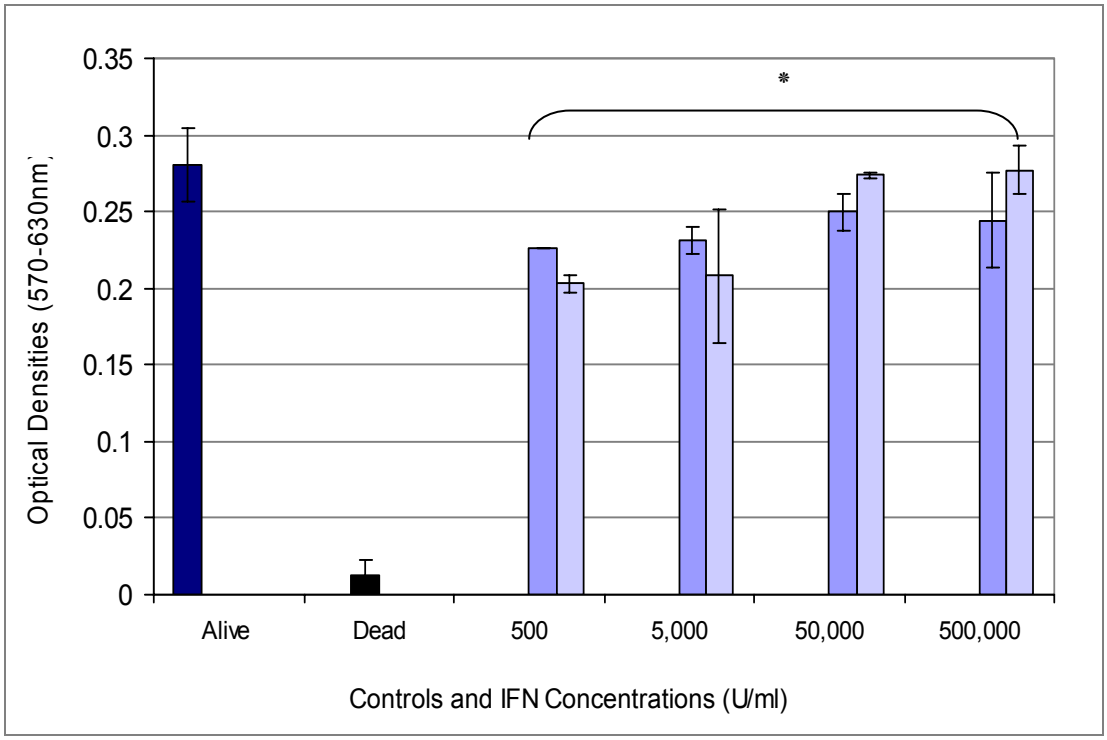

Figure 4.6. MTT-Assay for rHuIFN-α2b and rFeIFN-ω. Optical densities of tested IFN concentrations are compared towards controls ("alive" and "dead" cells). Asterisks indicate values, which are significantly different from "dead" control ($p<0.05$) but also significantly in accordance with "alive" control ($p<0.05$).

5. Discussion

In the present study, the effect of rFeIFN-ω and rHuIFN-α2b on FHV-1 replication was tested *in vitro*. Plaque formation of other origin (i.e. toxic effects, contamination with other cytolytic viruses) was excluded and FHV-1-involvement confirmed by IFA staining. Both interferons were shown to have antiviral effects and both are therefore useful for the treatment of FHV-1 infected cats. As hypothesized, treatment with rFeIFN-ω was more effective than treatment with the heterologous rHuIFN-α2b. The results showed for the first time that treatment efficacy for interferon cannot only be measured in terms of a reduction of the number of viral plaques but also in terms of a reduction of their size. Cytotoxic effects that potentially could have contributed to the anti-viral effect of the drugs were excluded by MTT-assay.

5.1. Experimental Results

The results demonstrated that both rFeIFN-ω and rHuIFN-α2b have antiviral effects on FHV-1 *in vitro*. The extent to which either rFeIFN-ω or rHuIFN-α2b were able to confer significant protection against FHV-1 was assessed by the (a) number of plaques and (b) the sizes of plaques in treated cultures compared to untreated controls.

There was some indication that the treatment with both rFeIFN-ω and rHuIFN-α2b resulted in a reduction of plaque number (Figure 4.2). However, the effect was found to be statistically significant only at the higher concentrations of rFeIFN-ω. A linear regression analysis showed that the size of the reduction increased with increasing concentration of rFeIFN-ω demonstrating that rFeIFN-ω has a dose dependent effect (Figure 4.3). None of the rHuIFN-α2b treatments resulted in a statistically significant plaque number reduction and there was no statistically detectable trend for the plaque reduction to be greater at higher concentrations (Figures 4.2 and 4.3).

It cannot be concluded from these results that there is no antiviral effect of rHuIFN-α2b on FHV-1 replication. It can only be concluded that if there is an antiviral effect the assay technique is not sufficiently sensitive to detect it. That there is likely to be a small antiviral effect is suggested by the finding that two of the tested concentrations were found to be close to statistically significant ($p > 0.05$ but less than 0.1). As described below, using plaque size reduction

rather than plaque number reduction as a measure of antiviral activity did provide evidence for an antiviral effect of rHuIFN-α2b.

The antiviral effect of rFeIFN-ω on plaque number was large enough to be detected by the assay technique, at least at higher concentrations (Figure 4.2). In agreement with reports by other authors, the *in vitro* antiviral activity of rFeIFN-ω was found to be dose-dependent (MOCHIZUKI ET AL., 1994). The most profound plaque reduction, obtained at the highest rFeIFN-ω concentration tested, was considerably less than that reported in previous studies (MOCHIZUKI ET AL., 1994; TRUYEN ET AL., 2001). However, there are major differences between the type of assays (e.g. treatments, viral input, cell line, anti-cellular effects) that influence the antiviral effect and contribute to the variation between those studies.

In general, the susceptibility of cell cultures to interferon has been found to vary considerably depending on cell-line and interferon type (WECK ET AL., 1981; FUERST ET AL., 1986). Biological effects such as initiation by activation of receptors and different binding-affinity may influence the mechanism of antiviral efficacy. In addition, the number of cellular receptors for IFN can vary considerably from cell-type to cell-type (10 - 2000/cell) (FLINT ET AL., 2000). TRUYEN and co-investigators (2001) reported that the treatment with rFeIFN-ω resulted in a less profound effect in CRFK-cells than observed in equally treated fcwf-cells: the fcwf-cell line used was found to be 10-times more sensitive to the rFeIFN-ω treatments than the CRFK-cells (TRUYEN ET AL., 2001). This may be attributable to a lower number of the IFN-receptor expression by CRFK-cells (TRUYEN ET AL., 2001).

Both MOCHIZUKI ET AL. (1994) and TRUYEN ET AL. (2001) used virus yield reduction assays for testing the effect of interferon on FHV-1 replication. Thus, the virus-yield reduction assay differs procedurally from the PR assay used in the current study: the former has a higher viral input, lacks an overlay media, involves freezing of the virus suspension and the assay procedure is of much longer duration. As is true for all biological assays, the exact concentration of an antiviral required to inhibit the replication of a virus depends on the infectivity input of the virus (MOI), the cell type used, the duration of the experiment, the kinetic of infection in that particular cell type and the sensitivity of the assay being used (SWIERKOSZ AND BIRON, 1995). It is to be expected that the yield reduction assay will result in more profound reduction due to the higher viral input. It is currently unknown whether clinical results are better correlated with the results obtained from the yield reduction assay or with the PR assay used here. This is a matter for further study. Thus, further empirical research is needed to determine why

differences existed between the antiviral effects of rFeIFN-ω reported here and those reported earlier.

The results using the plaque size measurements demonstrated statistically reliable plaque reduction relative to untreated controls with both rFeIFN-ω and rHuIFN-α2b at the higher concentrations examined (Figure 4.4). The plaque size reductions observed were similar for both rFeIFN-ω and rHuIFN-α2b (Figure 4.4). This demonstrates that rHuIFN-α2b did have an antiviral effect in the cell cultures used in the study reported, but this effect could only be reliably detected using the plaque size measure.

The host specificity of type I interferons is well known and maximal antiviral activity is described in homolog cells (FULTON AND BURGE, 1985; WEISS, 1989; WEISS ET AL. 1991). In contrast, previous observations of anti-FHV-1 activity in fcwf-cells or in feline lung cells have shown rHuIFN-α subtypes to inhibit FHV-1 replication *in vitro* (FULTON AND BURGE, 1985; WEISS, 1989).

None of the high-dose treatments of either rHuIFN-α2b or rFeIFN-ω caused significant cellular toxicity. Therefore, the anti-viral activity demonstrated by both interferons is not attributable to an *in vitro* effect on the cellular viability of CRFK-cells. As mentioned before (section 2.5) it has been suggested that CRFK-cells are less susceptible to IFNs in vitro. This may also explain that there was no cytotoxic effect measured in the MTT-Assay. However, the absence of cytotoxicity in *in vitro* tests does not necessarily exclude toxicity *in vivo*.

The results can be summarized as follows: rFeIFN-ω had a dose dependent antiviral effect that could be reliably detected at higher concentrations (> 50,000 U/ml) using both the plaque number and plaque size measurements. rHuIFN-α2b also had an antiviral effect but this could only be detected at high concentrations using the plaque size measure; there was no statistical evidence for a reduction in plaque number. As described earlier, the two measures of anti-viral activity (plaque number and plaque size) are likely to capture different aspects of the activity of the anti-viral agent. One action of IFNs is to inhibit viral replication within an infected cell. This action of the drug is expected to primarily affect plaque number. A second action of IFN is to protect an uninfected cell from infection by a virus. This action will affect the extent to which viral infection can spread from an infected cell to its neighbors and so is expected to primarily affect plaque sizes. This would mean that a somewhat similar effect of rHuIFN-α2b and rFeIFN-ω on plaque size reduction indicates a similar ability to protect uninfected cells against viral infection. The greater plaque number reduction observed with

rFeIFN-ω would indicate that this IFN has a greater ability to inhibit viral replication in infected cells.

There is currently no direct evidence that rHuIFN-α2b and rFeIFN-ω differ in the mechanism of their antiviral action and so the observed differences in their efficacy cannot, at this stage, be directly attributed to a difference in mechanism. However, since the two proteins differ in their molecular structure they may differ in binding affinity or cellular receptor specificity. This may result in a later stimulation of the induction of the antiviral state in culture. However, at this time too little is known to be able to say anything definitive concerning the differences observed in the current study.

5.2. Clinical Implications

The predictive value for clinical application based on *in vitro* studies is limited due to the different conditions *in vivo* and individual metabolic conditions of animal-patients. Nevertheless, the results of the present *in vitro* study suggest that either rFeIFN-ω or rHuIFN-α2b may be of prophylactic and therapeutic clinical value if given at high-doses early during FHV-1-infection. Use of rFeIFN-ω is likely to be the more effective.

The ability of the test-compounds to inhibit viral cell-to-cell spread may be relevant for clinical applications such as the topical treatment of FHV-1 induced dendritic keratitis. It is likely that inhibition of viral spread will act to restrict the development of dendritic ulcers. This possibility needs to be tested directly, not only because the study reported here was *in vitro* but also because the cell culture used was different to that of the intended clinical application (cornea cells).

A recent clinical study reported most profound clinical improvement of ocular FHV-1 manifestations following the topically treatment with rFeIFN-ω (VERNEUILE, 2004). The effects of the feline interferon were evaluated over a wide range of concentrations and it was interesting that the most profound improvement was observed at the same high-dose treatment (500,000 U/ml) that proved effective in the present study. Furthermore, high-dose treatment with the same concentration resulted in a prolonged biological activity of rFeIFN-ω, measured by the expression of Mx-proteins in cats (BRAECKLEIN ET AL., 2003). In both studies a dose-dependent response was observed (BRAECKLEIN ET AL., 2003; VERNEUIL, 2004). This indicates that the results from the test-systems used in the present study may apply to corneal cells *in vivo*.

Both interferons tested have immune modulating properties including the increase of the expression of class I MHC molecules and induce cellular anti-viral responses i.e. cytolytic activity of NK cells which inevitably result in a limited spread of progeny virus but also in loss of infected tissue. (FLINT ET AL., 2000; BIRON, 2001; ABBAS AND LICHTMAN, 2003). IFNs Thus, an enhanced effect of antiviral activity of these interferons may be expected *in vivo*.

In the question of value for the heterologous rHuIFN-α2b compound, there is the question how long the compound will be of clinical value as the continued efficacy of heterologous IFNs has been demonstrated to induce the formation of cytokine-specific neutralizing antibodies. Cats infected with established feline leukemia virus (FeLV)-acquired immunodeficiency syndrome (FAIDS), resistance to the HuIFN treatment due to neutralizing antibodies has been described previously (ZEIDNER ET AL., 1990).

5.3. Conclusions

This study was able to demonstrate anti-FHV-1 activity using high concentrations of rFeIFN-ω or rHuIFN-α2b *in vitro.* In addition, rFeIFN-ω was found to be more effective than rHuIFN-α2b, particularly in its ability to reduce plaque number. High-dose treatment with rFeIFN-ω or rHuIFN-α2b resulted in significant plaque size reduction indicating potential efficacy on reducing the dimensions of FHV-1 induced cytopathic lesions. This might be relevant for clinical application and it would be worthwhile to test for an effect for the treatment of FHV-1 infection in cornea cells.

Treatment with rFeIFN-ω has shown more profound effects in antiviral activity compared to rHuIFN-α2b and, in contrast to rHuIFN-α2b, it also offers the advantage of a homologous compound. This is consistent with the results obtained on *in vivo* activity, which have shown that high-dose IFN treatment shows good antiviral efficacy and clinical improvement. Therefore, there are indications that rFeIFN-ω may provide effective prophylactic and therapeutic treatment for FHV-1 infected cats.

6. Zusammenfassung

Das feline Herpesvirus-1 (FHV-1) ist ein weltweit bei Katzen vorkommendes DNA-Alphaherpesvirus, das neben den Oberflächenepithelien des Respirationstrakts auch die Konjunktiven infiziert. Der Erreger stellt eine der wichtigsten Ursachen des Katzenschnupfens dar und wird als häufigste Ätiologie bei der Konjunktivitis und auch der Keratits der Katze nachgewiesen. Vor allem bei Tieren, die in Gruppen gehaltenen werden, ist eine hohe Inzidenz (> 70 %) feststellbar. Im Anschluss an eine Primärinfektion entwickeln fast alle Katzen (> 80 %) einen Trägerstatus. In der Latenzphase persistiert das Virus hauptsächlich im Trigeminalganglion, aus dem es spontan oder durch eine Reihe anderer Ursachen (u.a. Stress, Cortisontherapie) zu einer Reaktivierung der Erkrankung und Virusausscheidung kommen kann. Eine Therapie der FHV-1 Infektion mit Virostatika (z.B. Aciclovir) ist sowohl systemisch als auch lokal möglich, aber umstritten, da sie in einigen Fällen nur geringe Wirkung zeigt, und die systemische Anwendung mit erheblichen toxischen Nebenwirkungen verbunden ist. Die erfolgreiche Behandlung, vor allem der bei persistierenden Infektionen wiederkehrenden okulären Manifestationen, stellt eine therapeutische Herausforderung dar.

In der Literatur wird die Anwendung humaner und feliner Interferone und ihre antivirale Wirkung gegen ausgewählte feline und canine Viren beschrieben (JAMESON & ESSEX, 1983; FULTON & BURGE, 1985; WEISS & TOIVIO-KINNUCAN, 1988; WEISS, 1989; WEISS & OOSTROM-RAM, 1990; TRUYEN ET AL., 2001). Bisher existieren aber nur zwei *in vitro* Studien, die auf eine vielversprechende Wirkung des rFeIFN-ω in der Behandlung der FHV-1 Infektion hinweisen (MOCHIZUKI ET AL., 1994; TRUYEN ET AL., 2001).

Es war Ziel dieser Studie, den antiviralen Effekt des humanen rHuIFN-α2b (Intron®-A) sowie des felinen rFeIFN-ω (Virbagen®-ω) auf die Replikation von FHV-1 *in vitro* zu untersuchen und miteinander zu vergleichen. Dabei sollte im Gegensatz zu bisherigen Untersuchungen (WEISS, 1989; MOCHIZUKI ET AL., 1994, TRUYEN ET AL., 2001) die Wirkung eines breiteren Spektrums (100 U/ml – 500,000 U/ml) von Interferon-Konzentrationen untersucht werden. Dieses Spektrum wurde gewählt, um beschriebene Konzentrationen zu wiederholen und um zu untersuchen, ob eine dosisabhängige Wirkung sowie eine eventuell optimale Dosis der Interferone vorlägen. Aufgrund der engen Spezies-Spezifität wurde bei der Behandlung mit dem homologen rFeIFN-ω im Vergleich zu dem heterologen rHuIFN-α2b ein deutlicherer Effekt erwartet. Der

Plaquereduktionstest sowie alle Zell- und Viruskultur-Techniken mussten dazu vor Beginn der Experimente etabliert werden. Ausserdem konnte erstmals gezeigt werden, dass die antivirale Wirksamkeit von Interferon auch anhand der Plaquegrösse quantifiziert werden kann. Um einen Zusammenhang zwischen einem antiviralen Effekt und einer zelltoxischen Wirkung beider Interferone auszuschliessen, wurde die Zellstoffwechselaktivität behandelter im Vergleich zu unbehandelter Zellkulturen in MTT-Assays untersucht.

Im Plaquereduktionstest wurde die Wirkung von jeweils 12 abgestuften Konzentrationen (100 U/ml bis 500.000 U/ml) pro Interferon (rFeIFN-ω, rHuIFN-α2b) in Duplikaten untersucht und sechsmal wiederholt. Dazu wurden CRFK-Zellen in 24-Zellkultur-Lochplatten ausgesät und über Nacht inkubiert. Die Zugabe der jeweiligen Interferonlösungen erfolgte sechs Stunden vor Virusinkubation und wurde über den weiteren Verlauf des Versuchs aufrechterhalten. Als Kontrolle dienten CRFK-Zellen, die jeweils an Stelle von Interferon phosphat-gepufferte Kochsalzlösung (PBS) erhielten, abgesehen davon aber den gleichen experimentellen Bedingungen ausgesetzt waren wie die Versuchsgruppen. Im Anschluss an die 72 stündige Inkubation wurden die Zellen fixiert, das Overlay-Medium wurde entfernt und die Zellrasen mit Kristallviolett gefärbt. Die Anzahl der Plaques (pro Vertiefung und Interferonkonzentration) konnte unter dem invertierten Lichtmikroskop gezählt und anschliessend ausgewertet werden. Die Erfassung einzelner Plaquesdurchmesser erfolgte mikroskopisch mit einem Meβokular.

Das MTT-Assay wurde in 96-Mikrotiterplatten mit CRFK–Zellen durchgeführt. Die Wirkung der Interferone wurde für jede Konzentration in acht Parallelansätzen (n=16) ermittelt und zweimal durchgeführt. Als Referenz dienten eine Lebend-kontrollgruppe, in der die Interferonbehandlung durch PBS ersetzt wurde und eine Totkontrollgruppe, in der die Zellen mit Ethanol abgetötet worden waren. Die Mikrotiterplatten wurden inkubiert und anschliessend wurde das Medium durch MTT-haltiges Medium ersetzt. Nach weiterer Inkubation wurde das Medium durch einer HCL/Isopronalol-Lösung ersetzt, um die Kristalle aufzulösen. Die optischen Dichten in den Vertiefungen wurden spektrophotometrisch gemessen. Anschliessend wurden die mittleren optischen Dichten errechnet und mit den Kontrollwerten verglichen.

Zur statistischen Auswertung der Experimente wurde eine ANOVA und der Dunnett-Test herangezogen. Eine signifikante Plaquezahlreduktion konnte nur in den mit rFeIFN-ω behandelten Zellkulturen festgestellt werden. In den Konzentrationen 100.000 U/ml und 500.000 U/ml ergab die Anzahl der Plaques

eine Reduktion um 54,7 % ($p<0,05$) bzw. um 59,8 % ($p<0,05$) gegenüber den unbehandelten Kontrollen. Ausserdem konnte eine statistisch signifikante Reduktion der Plaquegröße bei 100.000 U/ml von 47,5 %, bei 250.000 U/ml von 81 % und bei 500.000 U/ml von 70,5 % gegenüber den unbehandelten Kontrollen festgestellt werden. rHuIFN-α2b induzierte in keiner der untersuchten Konzentrationen eine signifikante Plaquezahlreduktion. Dagegen konnte jedoch eine signifikante Reduktion der Plaquegröße bei den Behandlungen mit 100.000 U/ml, 250.000 U/ml, und 500.000 U/ml um 56,4 %, bzw. um 75,7 %, und um 69,8 % nachgewiesen werden.

Die optischen Dichten für rFeIFN-ω und rHuIFN-α2b waren für alle gemessenen Konzentrationen signifikant ($p<0,05$) übereinstimmend mit den optischen Dichten der Lebendkontrollgruppen und signifikant höher ($p<0,05$) verglichen mit den Werten der Totkontrollgruppe. Damit ist gezeigt worden, dass sowohl rFeIFN-ω als auch rFeIFN-α2b keine signifikante zelltoxische Auswirkung auf den Zellstoffwechsel hatte.

Mit dieser Arbeit ist es im wesentlichen gelungen, die antivirale Wirkung von rFeIFN sowie von rHuIFN-α2b auf die Replikation von FHV-1 zu bestätigen. Eine Dosis-Wirkungsbeziehung des rFeIFN-ω, sowie statistisch signifikanter Reduktion der Plaquezahl und Plaquegröße konnte bei Behandlung mit hohen Konzentrationen (> 50.000 U/ml) von Interferon beobachtet werden. Mit der rHuIFN-α2b-Behandlung konnte keine statistisch signifikante Plaquezahlreduktion, aber eine signifikante Reduktion der Plaquegröße nach Applikation hoher Konzentrationen (> 50.000 U/ml) festgestellt werden.

Erst kürzlich wurde in zwei *in vivo* Studien von einer guten antiviralen Wirkung hoher rFeIFN-ω Konzentrationen berichtet, welche die Ergebnisse der vorliegenden Studie bestätigen (BRAECKLEIN ET AL., 2003; VERNEUIL, 2004). Somit liegt die Vermutung nahe, dass besonders mit dem rFeIFN-ω ein Präparat zur Verfügung steht, das bei prophylaktischer und kontinuierlicher Applikation im Verlauf einer Infektion in der Lage ist, die Ausbreitung von Nachkommenviren und damit die Ausdehnung der zytotoxischen Läsionen in infiziertem Gewebe in vitro einzuschränken.

7. Summary

Nicola Siebeck

Efficacy of rHuIFN-α2b and rFeIFN-ω on Feline Herpesvirus-1 Replication *in vitro*

Feline herpesvirus-1 (FHV-1)-infection, also known as feline viral rhinotracheitis (FVR) is distributed world wide in the cat population, with a high incidence in colony cats (>70%). FHV-1 typically infects and replicates in epithelial tissue of the upper respiratory tract and conjunctiva causing cytopathic lesions. The virus is recognized as one of the most important pathogens of feline upper respiratory tract infections, conjunctivitis and keratitis in cats. Following primary infection over 80% of cats are unable to eliminate the virus and develop a carrier state, with intermittent episodes of virus shedding. During latency, the virus persists most often in the trigeminal ganglion and the disease can be reactivated following stress and corticosteroid therapy.

Successful treatment, particularly of ocular manifestations associated with recrudescent infection such as dendritic ulcers remains difficult. Available antiviral drugs are virostatic and in particular, the systemic application is associated with severe toxic side effects. Human natural and recombinant interferons and feline interferons have been described in their use against a selection of feline and canine viruses (JAMESON and ESSEX, 1983; FULTON and BURGE, 1985; WEISS and TOIVIO-KINNUCAN, 1988; WEISS, 1989; WEISS and OOSTROM-RAM, 1990; TRUYEN et al., 2001). To date, only two *in vitro* studies indicate a potent antiviral activity of the recombinant FeIFN-ω against FHV-1 (MOCHIZUKI et al., 1994, TRUYEN et al., 2001).

The purpose of this study was to evaluate the antiviral efficacy of the recently available rFeIFN-ω (Virbagen®-ω) and the human rHuIFN-α2b (Intron®-A) on the replication of FHV-1 *in vitro*. A wider range of concentrations (100 U/ml – 500,000 U/ml) was studied than used in the previous studies (WEISS , 1989; MOCHIZUKI et al., 1994, TRUYEN et al., 2001) in order to repeat previously tested concentration and further, to determine whether the drugs have a dose-dependant response of antiviral activity and which concentration would be the most effective treatment. The species-specificity of type I interferons is well known and therefore, it was suggested that rFeIFN-ω would result in a more profound effect compared to the rHuIFN-α2b due to its homologous nature. In addition to cell and virus culture techniques, a methodology for the plaque reduction assay was established.

Furthermore, for the first time antiviral efficacy for interferon was additionally measured by plaque size reductions and is reported in this study.

An in vitro MTT-Assay was integrated in the experiment to exclude possible cytotoxic effects that could, in principle, contribute to the antiviral effects observed with either of the interferon treatments.

For the plaque reduction assay, confluent monolayers of Crandell feline kidney (CRFK)-cells were grown in 24-well cell culture plates. Cells were treated with either rFeIFN-ω (Virbagen® Omega) or rHuIFN-α2b (Intron®-A) across a set of serial dilutions (100 U/ml - 500,000 U/ml). Cells were treated six hours before addition of FHV-1, concurrent with addition of FHV-1 and each drug concentration was added to the overlay medium (2X DMEM 1:1 with 1.6% Agarose). The treatments were performed in duplicates including virus controls, which received PBS instead of either one of the interferons and assays were performed on six occasions. Following incubation of 72 hrs, the cells were fixed with formalin, overlay plugs were discarded and the remaining cell layers were stained with crystal violet. Plaque numbers were counted under an inverted microscope and Plaque diameters were measured using a reticule.

For the MTT-assay, 96-well culture plates were seeded with CRFK-cells. In each of the test wells (n=16) growth medium was supplemented with either rHuIFN-α2b or with rFeIFN-ω. Control wells (n=72) received growth medium with PBS instead of either one of the Interferons. Cell-death controls were treated with ethanol to obtain 100% cell death. After incubation, the medium was aspirated and replaced with medium containing the MTT-solution. Following another incubation period, the medium of each well was removed and replaced with a solubilization solution (0.1N HCL/Isopropanol). The plates were incubated for an additional five minutes to dissolve the crystals and the plates were read using a plate reader. The average optical densities were calculated for each dilution and compared with that of the positive control wells. A one-way ANOVA and Dunnett's test were used for the statistical analysis of all experiments.

A significant reduction of plaque numbers was observed for rFeIFN-ω at 100,000 U/ml with a plaque reduction of 54.7 % and at 500,000 U/ml with a plaque reduction of 59.8 %. Plaque sizes were significantly reduced by 47.5 % at 100,000 U/ml and by 81 % at 250,000 U/ml and 70.5% at 500,000 U/ml. Recombinant HuIFN-α2b treatment did not succeed to produce any significant plaque number reduction. However, significant plaque size reductions were

observed following treatment with 100,000 U/ml, 250,000 U/ml and 500,000 U/ml with reductions of 56 %, 75.7% and 69% respectively.

None of the high-dose treatments of either rHuIFN-α2b or rFeIFN-ω caused significant cellular toxicity in the MTT-Assay. Therefore, the antiviral activity demonstrated by both interferons is not attributable to an in vitro effect on the cellular viability of CRFK-cells.

In agreement with previous authors, this study was able to demonstrated that rFeIFN-ω and rHuIFN-α2b have inhibitory effects on the replication of FHV-1. For rFeIFN-ω the antiviral effect is dose-dependent and could be reliably detected at high concentrations (> 50,000 U/ml) using both the plaque number and plaque size measurements. Treatment with high concentrations of rHuIFN-α2b also resulted in an antiviral effect, which was only detected at using the plaque size measure; there was no statistical evidence for a reduction in the plaque number measurement. The significantly smaller plaque sizes in drug–treated cell cultures indicate that high-dose treatment with rFeIFN-ω or rHuIFN-α2b may have potential efficacy on reducing the dimensions of FHV-1 induced cytopathic lesions.

Treatment with rFeIFN-ω has shown more profound effects in antiviral activity compared to rHuIFN-α2b and, in contrast to rHuIFN-α2b, it also offers the advantage of a homologous compound. This is consistent with recently published results obtained on *in vivo* activity, which have demonstrated that high-dose treatment of rFeIFN-ω shows good antiviral efficacy and clinical improvement (VERNEUIL, 2004; BRAECKLEIN et al., 2003). Therefore, there are indications that rFeIFN-ω may provide effective prophylactic and therapeutic treatment for FHV-1 infected cats.

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9. Appendix

9.1. Media and Sera

- **Dulbecco's Modified Eagle Medium (D-MEM), (1 x)**
  high glucose, 1 x liquid with L-glutamine, with pyridoxine hydrochloride, without sodium pyruvate, Gibco Cat. # 11965-118

- **Dulbecco's Modified Eagle Medium (D-MEM), (10 x)**
  high glucose, 10 x powder without sodium bicarbonate and sodium pyruvate
  Gibco Cat. # 12100-046(1x)

- **Fetal Bovine Serum (FBS)**
  qualified Origin: United States Performance, Mycoplasma, virus and endotoxin tested, 500 ml, Lot Nr. 1180094, Gibco Cat. # 26140
  Sera were heat inactivated at 56°C for 30 min

9.2. Supplements and Reagents

- **Sodium Bicarbonate,** powder, cell culture tested, 500 g,
  Lot # 062K0176, Sigma Cat. # S5761

- **L-glutamine**, powder, cell culture tested, 25 g,
  Sigma Cat. # G-5763

- **Penicillin G and Streptomycin**, frozen, containing
  Sodium penicillin G 10,000 U/ml and Streptomycin sulfate 10 mg/ml
  Sigma Cat. # P0781

- **Amphotericin B** (Fungizone), 2,5µg/ml,
  powder, prepared with sterile water; Sigma Cat. # A9528

- **Dimethylsulphoxide** (DMSO), 5 x 5 ml Ampules,
  Sigma Cat. # D2650

- **Dulbecco's Phosphate Buffered Saline**, liquid, 500 ml,
  Sigma Cat. # W3500

- **Trypsin-EDTA Solution,** frozen, 0.25%, 0.53 mM EDTA
  Sigma Cat. # T4049, working solution: samples of 15 ml

- **Trypan Blue Solution**
  VWR Cat. # 11173, working solution: 0.03 % (w/v)

- **Crystal violet**
  Powder, solubilize with DI water
  CAS # 548-62-9, working solution: 0.01 % (w/v)

- **MTT Formazan**, powder
  1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, 57360-69-7,
  Sigma Cat. # M0032

- **Sea Plaque Agarose 100 G**
  American Bioanalytic Sea Plaque Agarose 100G
  Cat. # NC9664839 through Fisher Scientific, working solution/ gel: 1,6% (w/v)

9.3. Titration of Virus Suspensions

Example: Reed and Muench Method

Log of Virus dilution	Infected test units	Cumulative infected (A)	Cumulativ non-infected (B)	Ratio of A/ (A+B)	Percent infected
-1	10/10	10+8+3 =21	0	21/21	100%
-2	8/10	8+3 =11	2+0= 2	11/13	85 %
-3	3/10	3+0 =3	7+2 =9	3/12	25 %
-4	0/10	0	7+2+10 =19	0/19	0 %

Dilution that corresponds to 50 % lies somewhere between dilution 10^{-2} and dilution 10^{-3}

The proportionate distance between these two dilutions is calculated as follows:

(% positive above 50%)-50% **=** Proportionate distance

(%positive above 50%)-(%positive below 50%)

$$\frac{85\%-50\%}{85\%-25\%} = 0{,}58$$

The 50 % end point is calculated as follows:

As serial 10 fold dilutions where used, the log dilution factor is 1.0

(log dilution above 50%) + (proportionate distance x log dilution factor) = log ID_{50}

i.e (-2) + (0,58 x –1.0) = 1,42

Therefore $ID_{50} = 10^{-1,42}$

This is the endpoint dilution, i.e.the diltion that will infect 50% of the test units inoculated.The reciprocal of this number gives rise to the virus titer in terms of infectious doses per unit volume. If the inoculation of virus dilution was 0.1ml the titer of the virus suspension would therefore be:

$10^{1,42}$ $TCID_{50}$ x $0.1ml^{-1}$

= 10 x $10^{1,42}$ TCID ml^{-1}

= $10^{2,42}$ $TCID_{50}$ ml^{-1}

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11. Curriculum Vitae

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