Establishment of a Quiscence Herpes Simplex Type 1 Infection in L929 Fibroblasts and Neuro-2A Cells by a Nucleoside Analogue Acyclovir

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ESTABLISHMENT OF A QUIESCENCE HERPES SIMPLEX TYPE 1 INFECTION IN L929 FIBROBLASTS AND NEURO-2A CELLS BY A NUCLEOSIDE ANALogue ACYCLOVIR

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

NOURA SHAKLAWOON
MD., Misurata University, 2003

2013

Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY NOURA SHAKLAWOON ENTITLED Establishment of a quiescence HSV-1 infection in L929 fibroblasts and Neuro-2A cells by a nucleoside analogue acyclovir BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Herpes simplex type 1 (HSV-1) quiescent infection was established in L929 cells, murine fibroblasts, and in Neuro-2A (N2A) cells, mouse neuroblastoma, by treating them with the nucleoside analogue acyclovir (ACV) 24 hours before infection. Subsequent release of virus from the non-productive state was accomplished by treating the cells with the histone deacetylase inhibitor trichostatin A (TSA). Treatment of both L929 and N2A cell lines with ACV 24 hours before infection induced protection from HSV-1 cytopathic effect. A quiescent state was confirmed by absence of virus plaques when supernatant fluids from ACV-treated HSV-1 infected cultures were titered on Vero cell monolayers.

Acyclovir was maintained in the cell culture medium for one day before infection and two days post infection. Removal of ACV from the culture medium did not permit reactivation of virus as determined by virus plaque assays. Virus reactivation was confirmed in ACV- treated HSV-1 quiescent cells by examining cell lysates for virus plaque forming units. Four days post infection of ACV-treated cells, culture medium was removed and replaced with medium containing TSA. Supernatant fluids from cultures treated with TSA showed virus production in plaque forming assays on Vero cells. Media from both lytically infected cultures and TSA reactivated cultures contained productive virus; however, media from latently infected cultures did not show the presence of infectious virus. Treatment of L929 cells with ACV 2 hours after infection with HSV-1 induced a quiescent effect better than treatment of the cells with AVC 24 hours before
infection, because higher cell densities survived. As expected quantitative real-time polymerase chain reaction (qPCR) analysis of HSV-1 transcripts showed about one fold increase in Latency Associated Transcript (LAT) and a decrease in the lytic cycle infected cell protein (ICP0) in ACV-treated HSV-1 infected L929 cells at 16 hours post infection as compared with the untreated HSV-1 infected L929 cells. At the same time, lytic cycle infected cell protein (ICP27) showed a one and half fold increase in ACV-treated HSV-1 infected L929 cells compared with the untreated HSV-1 infected L929 cells. At 48 hours post infection, both infected cell proteins (ICP27 and ICP0) and LAT were observed at low levels in ACV-treated HSV-1 infected L929 cells compared with the lytically infected control cells. In N2A cells, LAT was noted at low level in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells at 16 hours post infection. At the same time, ICP0 decreased and ICP27 showed approximately one fold increase in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells. At 48 hours post infection, both lytic cycle transcripts (ICP27 and ICP0) and LAT were observed at low levels in ACV-treated HSV-1 infected N2A cells as compared with untreated HSV-1 infected control N2A cells. LAT was present but not significantly increased in both cell lines although they were latently infected and maintained with the ACV treatment.
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LIST OF ABBREVIATIONS

CPE = Cytopathic Effects
TSA = Trichostatin A
ACV = Acyclovir
IE = Immediate early
E = Early
L = Late
VP 16 = Virion protein
VRE = VP16-response elements
HCF-1 = Host cell factor-1
Oct = Octamer binding protein
STAT = Signal transduction and transcription
SOCS-1 = Suppressor of cytokine signaling -1
cAMP = Cyclic adenosine monophosphate
PKA = Protein kinase A
TK = Thymidine kinase
NGF = Nerve growth factor
IFN –α = interferon alpha
IFN-γ = Interferon gamma
ICP0 = Infected cell protein 0
ICP27 = Infected cell protein 27
ICP4 = Infected cell protein 4
LAT = Latency Associated Transcript
MOI = Multiplicity of Infection
PFU = Plaque forming unit
HSV-1 = Herpes simplex virus type – 1
CMV = Cytomegalovirus
TG = Trigeminal ganglia
TAP = Transporter associated with antigen presentation
VGNs = Vestibular ganglion neurons
SIRC = Statens seruminstitut rabbit cornea
I would like to thank Dr. Nancy Bigley for all of her guidance and support that she has given to me throughout my graduate study. I would also like to acknowledge and thank my program director, Dr. Barbara Hull and Dr. F. Javier Alvarez-Leefmans for their suggestions and contributions in my research.

I am also thankful to my family in Libya for their love and encouragement. Special thanks to my lab colleagues and friends for their help and support.
DEDICATION

I would like to dedicate my thesis project to my husband Hosen for all his encouragement and support. Also, I would like to dedicate this thesis to my lovely kids: Alhasna, Omar, Janna, and Rudaina. They are my inspiration.
INTRODUCTION

Herpes simplex virus (HSV) is a member of Herpesviridae family of large double stranded DNA viruses. Herpes simplex virus type 1 (HSV-1) and herpes simplex type 2 (HSV-2) are able to establish both lytic and latent infections. HSV is a neurotropic virus which can establish a latent infection within the sensory neurons in trigeminal ganglia (Nicoll et al., 2012). The mechanism by which HSV enters latent infection remains poorly understood. HSV-1 is usually associated with oral mucosa infections and encephalitis. HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates (Whitley and Roizman, 2001). About 90% of the population worldwide is infected with HSV-1 (Nicoll et al., 2012). The infection ranges from mild symptoms to death. Following primary HSV-1 replication at the oral mucosa, the virus infects the primary efferent of sensory neurons that innervate these tissues and reaches the nerve cell body located in sensory ganglia by retrograde microtubule-associated transport (Nicoll et al., 2012). Inside the cell body of sensory neurons, the virus causes either a lytic replication cycle or a latent infection. Most of the neurons will survive the infection and the virus will enter a latent cycle. However, some neurons will be destroyed because of the productive infection (Miller et al., 1998; Danaher et al., 1999b). During latency, the virus genome assumes a linear pattern which is transcriptionally repressed except for the region that encodes the latency-associated transcript (LAT) (Coleman et al., 2008). Exposure to heat, stress, ultraviolet light, or trauma, can cause spontaneous occasional reactivation of HSV-1 (Kuhn et al., 2012). The reactivated virus reenters the replication cycle forming
an infectious virus, which is transported anterograde back to the epithelial cells and begins re-infection and subsequent transmission (Danaher et al., 1999b).

Many stimuli have been shown to induce HSV-1 reactivation in vivo and in vitro. However, the molecular events of virus reactivation are not well known because of the absence of an effective murine model for in vivo and in vitro HSV-1 latency establishment and reactivation. Acyclovir (ACV), a nucleoside analogue, has been used to induce HSV-1 latency in vitro. A combination treatment of the human fetus lung fibroblasts (HFL-F) with ACV and interferon-α (INF-α) resulted in HSV-1 latency that continued after the removal of the drug (Scheck et al., 1986). However, Rong et al., 1988 showed that ACV induced HSV-1 latency in rabbit corneal cells (SIRC) and removal of the ACV led to reactivation of the virus. Interferon-γ (IFN-γ) plays a role in the cellular response to HSV-1. Frey et al., 2009 showed that IFN-γ can inhibit HSV-1 replication in L929 fibroblasts, but not in HEL-30 keratinocytes. The HEL-30 cells were resistant to IFN-γ suppression because of up regulation of the protein suppressor of cytokine signalling-1 (SOCS-1) in these cells. Histone modification is involved in viral gene expression during lytic and latent infection. Quiescently HSV-1 infected human fetal lung fibroblasts (MRC-5) showed deacetylated histones in their repressed genome structure. Genome de-repression by HSV-1 superinfection or ICP0 delivery by recombinant adenovirus resulted in re-enrichment of the viral DNA with acetylated histone (Coleman et al., 2008). In addition, histone deacetylase inhibitor, trichostatin A (TSA), induced HSV-1 production from quiescently infected neuronally differentiated pheochromocytoma (PC12) cells. Furthermore, the LAT gene was the only viral gene that accumulated in response to inhibition of deacetylation by using TSA. These observations indicate that LAT promoter is
transcriptionally active. LAT was not required for the reactivation process because LAT-deleted virus reactivated similarly to the wild type virus after TSA and heat stress application (Danaher et al., 2005).

**Hypothesis:**

Since acyclovir (ACV) interferes with HSV-1 replication by binding viral thymidine kinase and blocking viral DNA synthesis, ACV may render murine L929 fibroblasts and neuro-2A cells susceptible to HSV-1 induced latency. If latency can be established, HSV-1 will be reactivated following treatment with trichostatin A (TSA), a histone deacetylase inhibitor, which increases acetylation and relaxation of chromatin, permitting virus replication.
LITERATURE REVIEW

HSV-1 LYTIC INFECTION

Lytic HSV-1 infection occurs in epithelial cells. The virus attaches to heparan sulfate, cell surface membrane receptor, (Wudunn and Spear, 1989). Attachment of the virus to the host cell is facilitated by viral glycoproteins B and C (Rajcani and Durmanova, 2000). Glycoprotein D facilitates virus adsorption to the host cell and glycoproteins H and L are responsible for membrane penetration of the virus into the host cell (reviewed in Rajcani and Durmanova, 2000). Furthermore, Dingwell et al., 1995, demonstrated that glycoprotein E and I are responsible for HSV-1 spreading from one neuron to another neuron.

HSV-1 lytic infection involves ordered expression of several classes of viral genes that consist of structural and nonstructural components (Honess and Roizman, 1974). The cascade starts with expressing immediate early (IE) genes (α genes), followed by that of early (E) genes (β genes), DNA replication and late (L) genes (γ genes). The maximum rate of synthesis by the α genes occurs 3 to 4 hours post infection. The β genes are responsible for the highest rate of directing protein synthesis 5 to 7 hours post infection. The γ genes directing protein expression increases until 12 hours post infection. Use of the protein synthesis inhibitor cycloheximide confirmed that IE polypeptides expression occurs without prior viral protein synthesis, but E and L polypeptides expression needs preceding IE protein synthesis (Honess and Roizman, 1974).

The IE genes consist of infected cell proteins (ICP0, ICP4, ICP22, ICP27, and ICP47) and Us1.5, N-terminally truncated form of ICP22 (reviewed in Nicoll et al., 2012).
Wysocka and Herr, 2003 revealed that IE genes have VP16-response elements (VRE) within their promoters. The virion protein (VP16) is the main factor that activates transcription of these genes (Figure 1).

Following the entry of the virus to the cell, the viral capsid facilitates the release of viral DNA into the nucleus (Nicoll et al., 2012). In the cytoplasm, VP 16 binds host cell factor-1 (HCF-1) protein, which has a nuclear localization sequence, leading to the entry of VP 16 into the nucleus (Boissiere et al., 1999). Once in the nucleus, both VP 16 and HCF-1 proteins form a trimeric complex with another protein, Octamer binding protein-1 (Oct-1) on the IE promoter and start IE gene transcription (Wysocka and Herr, 2003).
Figure 1: HSV-1 lytic infection and gene expression (Adapted from Wysocka and Herr, 2003).

HSV-1 LATENT INFECTION

Lack of in vitro cell line model has limited our understanding to HSV latency and reactivation. A cell culture model would permit inspection of the events leading to latency
to a single cell line without the effect of immune response that would alter virus behavior in the cell (Danaher et al., 1999a). Latent infection is characterized by presence of non-replicating viral genome in the infected neuron from which the virus can be reactivated intermittently. The lytic infection genes are not expressed and the host cell survives the infection. The immune system cannot identify latently infected neurons. Latent herpes virus infection can be divided into three phases: establishment of latency, maintenance of latency, and reactivation from latency (Figure 2).

**Establishment of latency**

In the establishment phase, the virus enters the neuronal cell in which the viral genome remains transcriptionally quiescent. Thus, the integrity of the neuron is not compromised, as the cytopathic effect of the productive infection does not occur (Wagner and Bloom, 1997). During the establishment of latent infection, gene expression is limited to a gene located within the long repeat elements of the viral genome. Transcription of this gene results in generation of the latency-associated transcripts (LATs) (Zabolotny et al., 1997). Establishment of latency may result from the inability of IE genes to induce lytic infection. Marshall et al., 2000 showed that HSV-1 established latency in mice in the presence of impaired IE gene expression and that latency was not affected by restoration of VP16, ICP0, or ICP4 coding sequences. These observations suggest that the latency is increased when IE gene expression is inadequate to initiate the lytic infection. Latent HSV-1 presents in the nucleus of infected neurons (Nicoll et al., 2012).

During latency, the ability of VP 16 to initiate lytic gene expression may be
inhibited by a defect in VP 16 transport from nerve endings to the neuronal cell body, or
due to the presence of this protein in reduced amounts in the neurons (reviewed in Miller
et al., 1998).

In addition, two competitive inhibitors (transcription factors) for VP 16 were
demonstrated: the octamer-binding protein (Oct-2) (Lillycrop et al., 1991) and N-Oct3
(Hagmann et al., 1995). These two factors are present in the sensory neurons and they
compete with VP 16 for the binding of α gene promoter. Another factor for the establish-
ment of latency in sensory neurons is failure of VP 16 to form a complex with HCF-1. In
sensory neurons, HCF-1 protein is present in the Golgi apparatus in the cytoplasm instead
of the nucleus. Upon reactivation of HSV-1, the HCF-1 protein moves to the nucleus
(Kolb and Kristie, 2008).

Octamer binding protein-1 (Oct-1) is present in almost all cell types including
proliferating cells of neuronal origin, but it is down regulated in mature non-dividing neu-
rons (Lakin et al., 1995), contributing to the failure of VP 16 to form a complex which is
important for IE gene transcription during lytic cycle.

Maintenance of latency

During the maintenance phase of latency, a reservoir of the viral genomes should
be increased on reactivation. To date, no viral gene expression appears to be needed for
latency maintenance in sensory ganglion (Wagner and Bloom, 1997; Miller et al., 1998).

Wilcox and Johnson, 1988 revealed that nerve growth factor (NGF) is essential
for HSV-1 latency maintenance in sympathetic ganglion neurons treated with acyclovir
(ACV) in vitro. They showed that NGF withdrawal caused reactivation of these neurons.
HSV-1 latency was induced by the using of ACV in these cells, although the role of NGF in latency maintenance is controversial.

**Reactivation from latency**

Reactivation of HSV-1 from latency includes a shift from quiescence to virus replication state within neurons of sensory ganglion. In humans, HSV-1 reactivation can be spontaneous or as a result of exposure to UV irradiation, emotional stress, fever, or immune suppression. Reactivation causes shedding of the virus transported through neuronal axons to the epithelial cells where the virus can replicate and start a lytic cycle.

In culture, researchers have found different stimuli that can reactivate HSV-1 from latency. Nerve growth factor deprivation from neurons resulted in HSV-1 reactivation from latency (Wilcox and Johnson, 1988). Heat stress can reactivate HSV-1 from latency in PC12 cells (Danaher et al., 1999b; Miller et al., 2003; Danaher et al., 2005). Histone deacetylase inhibitors Trichostatin A (TSA) (Danaher et al., 2005; Roehm et al., 2011; Kuhn et al., 2012) or sodium butyrate (Danaher et al., 2005) can induce HSV-1 production from a quiescence state in neuronal cells by acceleration of HSV-1 genome hyperacetylation and relaxation. Danaher et al., 2005 examined the viral gene response to TSA and found only LAT accumulated in response to TSA reactivation. However, they showed that LAT is not necessary for this reactivation because the LAT mutant HSV-1 reactivated as the wild type virus in the neuronal cells. Forskolin, which increases the cyclic adenosine monophosphate (cAMP) signaling, can induce HSV-1 reactivation in neuronal cell line (Danaher et al., 1999a; Miller et al., 2003; Danaher et al., 2005). Activation of adenylate cyclase causes elevation of cAMP in the cells. Then cAMP binds protein kinase A (PKA) that can release catalytic subunits leading to phosphorylation of specific transcrip-
tion factors. Increased levels of cAMP may contribute to HSV-1 reactivation because LAT promoter is cAMP responsive. Phosphorylation may also include transcription factors needed for ICP0 and ICP4 expression (reviewed in Miller et al., 1998). HSV-1 can be reactivated by superinfection with cytomegalovirus (CMV) (Scheck et al., 1986) or adenovirus vector encoding exogenous HSV-1 ICP0 which de-presses the quiescent viral genome (McMahon and Walsh, 2008; Coleman et al., 2008).

In experimental animals, hyperthermia was efficiently induced HSV-1 reactivation from latently infected mice (Sawtell and Thompson, 1992). HSV-1 reactivation was limited to few neurons following stimulation of the whole ganglion.
Figure 2:  HSV-1 lytic and latent infection (adapted from Miller et al., 1998).

FUNCTION OF LAT

Transcription of LAT results in production of an 8.3 kb ‘minor LAT’ primary transcript, which is then spliced to produce a stable 2.0 kb intronic and an unstable 6.3 kb exonic RNA (Figure 3). The 2.0 kb intron is furthermore spliced to produce an additional stable 1.5 kb intron. The last two RNA species are named as the “major LATs”. The minor and major reflect the abundance of these transcripts in the experimental systems (Zabolotny et al., 1997). A protein encoded by the LAT has not been detected
(Doerig et al., 1991; Umbach et al., 2008). However, Doerig et al., 1991 showed that LAA, latency-associated antigen, was present in primary neurons latently infected in vitro. This antigen was not present in neurons with mock infection, in Vero cells with lytic infection, or in neurons with latent infection by a mutant virus carrying a deletion in the LAT gene. The function of this protein was not clear. They found that LAT mutant virus established a latent infection and reactivated from explanted trigeminal ganglia of mice as the wild type virus. These observations suggest that LAA may stay inactive during the latent infection and be activated by extracellular stimuli that trigger virus reactivation (Doerig et al., 1991).

To date, the function of LAT is still not well understood. It is surprising that LAT expression is not an absolute indication for latency establishment (Javier et al., 1988), as LAT defective HSV-1 can establish latent infection in mice (Block et al., 1990). In contrast, Thompson and Sawtell in 1997 revealed that the LAT gene has a role in establishment of latency, but LAT has no direct role in the HSV-1 reactivation. They found that about 30% of the TG neurons in mice infected with LAT+ HSV-1 harbored latent virus. However, 10% of the neurons in mice infected with LAT-null viruses were positive for HSV-1 DNA. To confirm the role of LAT deletion in the decreased frequency of latency establishment, they rescued the LAT gene by repair of the LAT genome and by expression of the 2.3-kb ApaI fragment that contains the major LAT sequences. The rescued LAT gene showed wild-type establishment and reactivation. HSV-1 with mutated IE gene can establish latency (Marshall et al., 2000).

The LAT exonic region functions as a primary microRNA (miRNA) precursor that encodes four different miRNAs in HSV-1 infected cells (Umbach et al., 2008). The
miR-H2-3p is transcribed as antisense to ICP0. The miR-H2-3p is able to reduce ICP0 protein expression, but does not significantly affect ICP0 mRNA levels. The miR-H6 inhibits expression of ICP4. These results may explain the ability of LAT to induce latency (Umbach et al., 2008; Umbach et al., 2009). These miRNA precursors act as post-transcriptional regulators of viral gene expression to help in induction and maintenance of HSV-1 latency in the host cell.

Figure 3: The LAT region of HSV-1 genome (Adapted from Nicoll et al., 2012).
IMMUNE RESPONSE TO HSV-1

It is important to understand the mechanisms that control the latency of HSV-1 because its reactivation from latency is associated with pathological disease due to shedding of the reactivated virus from the sensory ganglia (Knickelbein et al., 2008). Knickelbein et al., 2008 found that CD8⁺ T cells can inactivate HSV-1 without inducing neuronal apoptosis. They showed that CD8⁺ T cell lytic granules, containing granzyme B, can destroy the HSV-1 immediate early protein, ICP4, which acts as transactivator of β genes required for viral DNA replication.

Theil et al., 2003 have shown that HSV-1 latency is accompanied by chronic inflammatory response without neuronal damage. Latently HSV-1 infected trigeminal ganglia (TG) showed infiltration with CD3⁺ and CD8⁺ T cells, CD68-positive macrophages, interferon-γ, tumor necrosis factor-α, IP-10, and RANTES. Researchers suggested that the presence of the immune cells and elevated levels of cytokines inside the neurons of latently infected trigeminal ganglia may explain why immunosuppressive drugs cause viral reactivation.

CD8⁺ T cells and macrophages and their cytokine, tumor necrosis factor, have a role in maintaining HSV-1 latent in the trigeminal ganglia. However, natural killer cells and γδ T cells and their cytokine gamma interferon play a role in preventing viral replication during the lytic infection (Liu et al., 1996).

Feldman et al., 2002 explained the immune cells infiltration in latently infected trigeminal ganglia by spontaneous reactivation of some neurons leading to expression of HSV-1 lytic cycle transcripts. Because of absence of detectable virus in latently infected trigeminal ganglia, they called this situation a spontaneous molecular reactivation.
T lymphocytes, CD8⁺ T cells, have an immunological role against viral infection. CD8⁺ T cells can recognize viral antigens produced by viral infected cells and attack these cells. HSV-1 has developed different ways to avoid the immune response. HSV-1 is able to escape the immune response of the CD8⁺ T cells in the neuronal tissue by expression of ICP 47 protein, an HSV-1 immediate protein (Goldsmith et al., 1998). The ICP47 inhibits HSV-1 infected cell recognition by CD8⁺ T by inhibiting the transporter associated with antigen presentation (TAP). The ICP 47 blocks the expression of HSV-1 antigens on the MHC I on the surface of viral infected cells. HSV-1 with ICP47 mutation is less neurovirulent than wild-type HSV-1 in mice, but both of them are able to replicate normally in epithelial tissues. The ICP47 mutant HSV-1 showed reduced neurovirulence in immunologically normal mice and T cell–deficient mice after reconstitution with CD8⁺ T cells.

Researchers have shown another way by which HSV-1 can evade the immune response by SOCS-1 expression, a suppressor of cytokine signaling-1 (Frey et al., 2009). They found that HSV-1 was resistant to antiviral effect of IFN-γ in keratinocytes. HSV-1 infected keratinocytes exhibited high levels of SOCS-1 mRNA and protein. HSV-1 prevented IFN-γ signaling by inhibition of STAT1α activation. ICP0 has a role in the activation of the host gene SOCS-1 (reviewed in Frey et al., 2009).

MODELS FOR STUDYING HSV-1 LATENCY

Animal models rabbits, guinea pigs, and mice have been used to study HSV latency and reactivation. These animal models gave us the vast majority of our understanding of HSV latency and reactivation. However, the use of these models is
limited because the pattern of infection is strain specific, the neurovirulence of HSV in
these animals can be altered by gene manipulation to understand viral gene relationship
and the inadequate number of neurons latently infected with the virus (Wagner and
Bloom, 1997).

The mouse model is most commonly used to study HSV-1 latency. After
peripheral inoculation of the virus into the cornea or footpad of the mouse, the virus
replicated in the epithelial cells then transported by axons to the regional sensory
ganglion. Three to ten days post infection the virus can be discovered in sensory ganglion.
The immune system then clears the lytic infection and latent infection is established
(Nicoll et al., 2012). Reactivation usually does not occur spontaneously, and it can be
carried out by in vivo induction of reactivation by hyperthermia (Sawtell and Thompson,
1992) or by sensory ganglion explantation into cell culture (Kuhn et al., 2012; Devi-Rao
et al., 1994; Camarena et al., 2010).

Rabbit corneal inoculation has also been used as an animal model. The virus
reactivation can be spontaneous or can be induced. In vivo induction of reactivation can
be conducted by application of transcorneal iontophoresis of epinephrine (Danaher et al.,
2005) or intravenous injection of cyclophosphamide followed with intravenous injection
of dexamethasone (Miller et al., 2003).

In vitro, cell culture models are superior to the animal models because they allow
observing the virus at the cellular level without the effect of immune response and it is
easier to deal with cells than with whole animals. Rat pheochromocytoma (PC12)
neuronally differentiated cells have been used as a model for HSV-1 latency
establishment with ACV (Danaher et al., 1999a; Miller et al., 2003; Danaher et al., 2005;
Danaher et al., 1999b). Kuhn et al., 2012 used geniculate ganglion neurons as a model for latency induction with ACV, and they used HSV-1 GFP-tagged DNA where GFP-positive cells detected by fluorescent microscopy. In cell culture models, latent virus can be confirmed by absence of productive virus from plaque assays of culture media on Vero cells. However, lytically infected or reactivated cultures show infectious virus and plaque formation. Coleman et al., 2008 used Human Fetal Lung Fibroblast Cells (MRC-5 Line) as latency model. Latency was established by infecting the cells with replication defective HSV-1 containing IE gene mutation. Reactivation was induced by superinfection with HSV-2 or ICP0 delivery by recombinant adenovirus vector. In culture models in vitro, the viral genome was found depressed and circular during latency as is found in vivo. However, LAT expression and reactivation have been seen only in neuronal cells. On the other hand, Scheck et al., 1986 have shown that in vitro HSV-1 latency can be established in human fetus lung fibroblast (HFL-F) cell line by using ACV and human leukocyte interferon (IFN-α). Virus reactivation was achieved by human cytomegalovirus superinfection or by reducing the incubation temperature from 40.5°C to 37°C.

**ACV BIOCHEMISTRY AND MECHANISM OF ACTION**

Acyclovir, 9-(2-hydroxyethoxymethyl) guanine, is acyclic purine nucleoside analogue (Figure 4) (Elion, 1982).
Acyclovir has potent inhibitory and selectivity for herpes viruses, especially herpes simplex viruses types 1 and 2 and varicella zoster virus (Elion, 1982). The selectivity of this drug to herpes viruses is due to the ability of these viruses to activate the drug by viral thymidine kinase (TK) to acyclovir mono phosphate. Acyclovir monophosphate is then converted to a triphosphate by cellular enzymes. Acyclo-GTP binds strongly to herpes virus DNA polymerases than to the cellular DNA polymerases and terminates DNA synthesis (Figure 5). Acyclo-GTP persisted in HSV-infected cells for many hours after acyclovir removal from the medium (Elion, 1982; Furman et al., 1981). In addition, (Furman et al., 1981) showed that Acyclo-GTP formation occurs even in the uninfected cells, but the enzymes responsible for first ACV phosphorylation are unknown.
TSA MODE OF ACTION

Trichostatin A (7-[4-(dimethylamino) phenyl]-N-hydroxy-4, 6-dimethyl-7-oxohepta-2, 4-dienamide) (TSA) is an antifungal antibiotic. Yoshida et al., 1990 showed that TSA can reversibly suppress cell cycle at G1 and G2 phases of the normal mouse diploid fibroblasts. It causes hyper acetylation of histones by inhibition of histone deacetylases. They found that TSA did not interfere with activity of other enzymes such as protein kinase, protein phosphatase, DNA topoisomerase, and calmodulin in vitro. Thus it can be used for biological analysis without side effects.
Trichostatin A has selectivity to inhibit class I and II HDACs (Dokmanovic et al., 2007). Both class I and class II deacetylases have zinc in their catalytic sites to which the TSA can bind and inhibit these enzymes. However, class III HDACs have sirtuin proteins in the catalytic site and are not inhibited by TSA. Histone deacetylase inhibitors cause hyperacetylation of histones, leading to alteration of gene expression. TSA interferes with the removal of acetyl group from histones leading to changing the ability of DNA transcription factors to access DNA in the chromatin structure.
MATERIALS AND METHODS

Cell culture

Cell lines used in this study were: L929 fibroblasts (CCL-1, ATCC) derived from C3H mouse connective tissue, N2A cells (CCL-131, ATCC) derived from mouse neuroblastoma cells, and Vero cells (CCL-81, ATCC) derived from the kidneys of African Green Monkeys. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamicin solution 50 μg /ml of the medium. Cells were grown on 100 mm² (L929 and Vero cells) and 60 mm² (N2A cells) tissue culture dishes and incubated in a humidified incubator at 37°C and 5% CO2. The N2A cells were not differentiated. Cell culture dishes and the culture medium were purchased from Fischer Scientific, Pittsburgh, PA.

Virus

Herpes simplex virus-1 (Syn 17+ strain) (obtained initially from Dr. Nancy Sawtell, Children’s Hospital Medical Center, Cincinnati, OH) was propagated in Vero cells. The Vero cells were grown in 100 mm² culture dishes as a monolayer and, at 90-100% confluency, cells were infected with HSV-1 at 0.1 MOI. Cytopathic effect (cells rounding and detaching from the bottom of the dish) appeared by microscopic examination 3-5 days post-infection. Then, the cells were harvested and the medium was stored as virus stock in 200 μl aliquots at 80°C. Plaque assay was performed using Vero cells to determine the virus plaque forming unit per milliliter (PFU).
Nucleoside analogue agent

Acyclovir (Sigma; St. Louis, MO) was purchased as a powder. A stock solution of 400 µg/ml was prepared in DMEM medium, supplemented with 2% FCS and maintained at 2-8°C for 2 weeks. Additional dilutions were made prior to use.

Histone deacetylase inhibitor

Trichostatin A (Sigma; St. Louis, MO) was purchased as a solution in DMSO and stored at -20°C. Dilutions were freshly made in the culture medium prior to use.

HSV-1 replication suppression

L929 cells

ACV treatment before infection (protocol 1)

L929 cells were cultured in 24 well plates at a seeding density of 2.5x10^4 to 3x10^4 in each well and incubated overnight. The next day, at 70-80% confluency, cells were treated with ACV at different doses (40, 20, and 10 µg/ml of medium) 24 hours before infection (Danaher et al., 2005). At 100% confluency, HSV-1 at 0.1 MOI was added onto L929 cell monolayer and incubated for 2 hours at 37°C. Then, the virus inoculum was aspirated and the medium containing ACV was added to the plates. The plates were incubated at 37°C in 5% CO2 for 48 hours (Frey et al., 2009). Untreated L929 cells (control cells), HSV-1 infected non ACV suppressed cultures (positive infection control), and ACV suppressed non-infected cultures (toxicity control) were maintained for each experiment (Rong et al., 1988). At 48 hours post infection, the cytopathic effect of HSV-1 was determined: cells were rinsed with PBS and fixed with 4% formaldehyde for 10 minutes,
then stained with 0.5% crystal violet for 5-7 minutes. The cells were then rinsed with water and allowed to dry at room temperature overnight. Plates were scanned on an HP Scan Jet 5300C. Pixel densities of the scanned plates were determined by using the NIH Image J program (Frey et al., 2009).

**ACV treatment after infection (protocol 2)**

The L929 cells were plated in 24 well plates in 10% DMEM and cultured overnight. The following day, cells were infected with HSV-1 at 0.1 MOI. After 2 hours, the residual virus was removed and the cells were incubated in culture medium containing ACV for 48 hours (Rong et al., 1988). The cytopathic effect was analyzed as discussed in protocol 1.

**N2A cells**

N2A cells were plated in 24 well plates at a seeding density of 2x10^4 to 2.5x10^4 to each well. At 40-50% confluency, cells were treated with ACV at different doses (20, 10, and 5 µg/ml of medium) 24 hours before infection. After 24 hours, cells were infected with the virus for 2 hours at an MOI of 0.1 at 37°C. Then, the virus was removed and replaced with culture medium containing ACV for 48 hours. After 48 hours, the cytopathic effect was examined as in the L929 cells.
Detection of Latency

Latent infection (lack of infectious virus in culture medium) of ACV-treated HSV-1 infected L929 and N2A cells at 48 hours post infection was confirmed by using supernatant fluids from ACV-treated HSV-1 infected cultures and titered on Vero cell monolayers (Kuhn et al., 2012).

I. Vero cells were grown in 24 well plates. At 90-100% confluency, the culture medium was removed and replaced with 250 µl of supernatant fluids from ACV-treated HSV-1 infected L929 cultures in each well and incubated for 1 hour for adsorption. After 1 hour, the supernatant was replaced with culture medium containing methylcellulose and incubated at 37°C in a 5% CO2 for 48 hours when they were checked for plaque formation (Shao-Chung et al., 2010).

II. For confirmation of the maintenance of latency, ACV was removed from the ACV-treated HSV-1 infected L929 and N2A cultures at 48 hours post-infection. Then, the cells were incubated in DMEM medium with 10% FCS for an additional 48 hours. At 48 hours, cultures were examined for the presence of productive virus using culture supernatant fluids in plaque forming assays on Vero cells.

HSV-1 reactivation

L929 and N2A cell lysates

Cells were protected from the cytopathic effect of HSV-1 by ACV treatment. To examine if the virus was silenced, cell lysates from the ACV-treated HSV-1 infected cultures were examined for PFUs. Cells were grown, treated, and infected as per HSV-1 suppression
experiment. At 48 hours post infection, ACV-treated HSV-1 infected cells were collected and stored at -80°C. Virus was released from ACV-treated HSV-1 infected cells by formation of lysates collected after two freeze-thaw cycles (Devi-Rao et al., 1994). Vero cells were plated in 24 well plates. At 90-100% confluency of the Vero cells, the lysate was added on the Vero cell monolayer for adsorption for 2 hours at 37°C. The lysate was then aspirated and replaced with culture medium containing methyl cellulose. Plates were incubated at 37°C in a 5% CO2 for 2-3 days and checked daily for the HSV-1 cytopathic effect (plaque formation).

**Trichostatin A**

L929 cells and N2A cells were grown in 24 well plates overnight. The following day, the cells were treated with ACV for 24 hours before infection. HSV-1 at 0.1 MOI was inoculated onto confluent cells and adsorbed for 2 hours at 37°C. The virus inoculum was then aspirated and replaced with DMEM containing ACV for 48 hours. At 48 hours post infection, the ACV containing culture medium was removed and the cells were incubated in DMED with 10% FCS for an additional 48 hours. Quiescent cultures (free of detectable infectious virus in culture supernatant fluids) were induced by TSA treatment using different doses (25 and 50 ng/ml of medium) to activate the virus. At 4 days post infection, cultures were induced by incubation in DMED with 10% FCS containing TSA and kept at 37°C in a 5% CO2 for 48 hours. After 48 hours, virus release from TSA treated cells was examined by titering culture medium on Vero cells (Danaher et al., 2005; Kuhn et al., 2012).
**Quantitative real time PCR for viral gene transcripts**

L929 cells and N2A cells were plated in 60 mm Petri dishes at seeding density of 1-1.5x10^5. Cells were maintained in DMEM supplemented with 10% FCS. Cells were treated with ACV 24 hours before infection. Cells were infected with HSV-1 at an MOI of 1 and maintained in DMEM with 2% FCS containing ACV. Cultures were maintained in ACV 1 day before infection and 48 hours post infection.

**RNA extraction**

RNA was extracted at different time periods using RNeasy Mini Kit (Qiagen) as per manufacturer’s instructions. RNA was extracted 16 hours post infection and at 48 hours post infection in presence of ACV. Positive virus control (lytic infection) was run at both time periods of RNA extractions. 50 µl of RNA was eluted. The concentration of RNA was determined using nanodrop. A total of 10 µg of the eluted RNA was treated with DNase I to eliminate DNA contamination using DNase I Amplification Grade (Invitrogen) per manufacturer’s instructions. Then, 1 µg of the DNase I treated RNA was reverse transcribed to cDNA using Qscript cDNA Synthesis Kit (Quanta) as recommended.

**Quantitative real-time PCR (qPCR)**

The relative quantity of cDNA synthesized for HSV-1 transcripts was determined using qPCR. The control gene was GAPDH (housekeeping gene), and viral genes were ICP27, ICP0, and major LAT. Primers and probes were used as discussed by Danaher et al., 2005 (Table 1). Primers and probes were purchased from Integrated DNA Technology.
(IDT) as Prime Time Std qPCR Assay (mixed primers and probes). Probes were labeled at the 5’ with the reporter fluorochrome, 6-carboxy-fluorescein (6-FAM), and at the 3’ end with Iowa Black FQ. Each sample was used in triplicate. Real-time PCR was performed on Applied Biosystems 7900 HT. 20 µl reaction volume was used. The final concentration of 10X Assay was 2 µl of 10X Prime Time Assay, 10 µl of 2X Master Mix (Thermo Scientific Maxima Probe/ROX qPCR Master Mix 2X), and 8 µl of cDNA and RNase-free water. Data analysis was performed using Applied Biosystems SDS 2.4 software.

**Statistical analysis**

Statistical significance was calculated using a paired t-test (SigmaPlot 12.0); all experiments were repeated three times. Standard error of mean was measured to all the samples.
RESULTS

Cytopathic inhibition assay for ACV treated HSV-1 infected L929 cells

The cytopathic inhibition assay was used to determine which concentration of ACV to use for pretreating L929 cells to induce HSV-1 latency after infection with an MOI of 0.1. L929 cells treated with 10 μg /ml of ACV were protected from the cytopathic effect of HSV-1. No plaques were visible in ACV-treated HSV-1 infected cells in comparison with untreated infected control cells (Figure 6). Higher concentrations of ACV (40, 20 μg /ml) were protective against the cytopathic effect of HSV-1, but were toxic to L929 cells. Eighty percent of the cells treated with 10 μg /ml of ACV survived. However, cells treated with 40 μg /ml and 20 μg /ml showed survival levels of 41% and 56%, respectively (Figure 7). Cell survival was determined by pixel density measurement using the image J program.
Figure 6: HSV-1 cytopathic effect at different concentrations of ACV added to L929 cells before infection. Virus plaques were seen in infected untreated control cells (wells 2). There were no plaques in ACV-treated HSV-1 infected cells (wells 8). High ACV concentrations were toxic as noticed by the decrease in the cell density (wells 6 and 7).

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</tr>
<tr>
<td>2</td>
<td>L929 + HSV-1</td>
</tr>
<tr>
<td>3</td>
<td>L929 + 40 (μg/ml) ACV</td>
</tr>
<tr>
<td>4</td>
<td>L929 + 20 (μg/ml) ACV</td>
</tr>
<tr>
<td>5</td>
<td>L929 + 10 (μg/ml) ACV</td>
</tr>
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</tr>
<tr>
<td>7</td>
<td>L929 + HSV-1 + 20 (μg/ml) ACV</td>
</tr>
<tr>
<td>8</td>
<td>L929 + HSV-1 + 10 (μg/ml) ACV</td>
</tr>
</tbody>
</table>
Figure 7: HSV-1 cytopathic effect at different concentrations of ACV added to L929 cells before infection. Higher concentrations of ACV have less cell survival after HSV-1 infection. Cell survival was determined by measuring % pixel density of scanned images. The pixel density was normalized relative to untreated, uninfected L929 control cells. ACV (10 µg/ml) treated HSV-1 infected cells have higher survival relative to untreated infected control cells; P<0.001 by ANOVA.
Comparison of ACV treatment before and after HSV-1 infection

Treatment of L929 cells with ACV 2 hours after infection resulted in better cell survival compared with cells treated with ACV 24 hours before infection (Figure 8). Cell survival was 90% using 10 µg/ml of ACV after infection and 80% when using ACV before infection. Using a higher concentration of ACV (20 µg/ml) after infection showed better survival (82%) compared with same dose before infection (58%), (Figure 9). The explanation for this effect might reflect the time of exposure to ACV, three days in case of ACV treatment before infection and only two days in case of ACV treatment after infection. Examination of HSV-1 cytopathic effects was carried out at two days post infection in both situations. For the subsequent experiments, ACV treatment 24 hours before infection was used because ACV treatment after infection inhibited virus replication completely.
Figure 8: Comparison between HSV-1 cytopathic effects at L929 cells treated with ACV before and after infection. Virus plaques were seen in infected untreated control cells (wells 2). There were no plaques in ACV treated HSV-1 infected cells (wells 5-8). ACV treatment after infection showed better protection from HSV-1 CPE as noticed by the increase in the cell density (wells 6 and 8).
Figure 9: Comparison of HSV-1 cytopathic effects at L929 cells treated with ACV before and after infection. ACV treatment after infection has higher cell survival. Cell survival was determined by measuring % pixel density of scanned images. The pixel density was normalized relative to untreated, uninfected L929 control cells. HSV-1 infected L929 cells treated with ACV (10 µg/ml) 2 hours after infection showed greater survival relative to L929 cells treated with ACV before infection; P=0.015 by ANOVA. A=after infection. B=before infection.
**HSV-1 latency in L929 cells**

Culture media samples from L929 cells treated 24 hours before HSV-1 infection were collected at 48 hours post infection and applied to Vero cell monolayers; no plaques formed. This suggested that the virus was silenced in these cells (Figure 10, a). Removal of ACV from the culture medium did not cause reactivation of the virus. At 48 hours post infection, the culture medium containing ACV was removed and replaced with culture medium without ACV, and cells were incubated for an additional 48 hours. At 4 days post infection, culture media were collected and applied to Vero cells; there was no plaque formation (Figure 11, a), indicating that the virus remained silenced after ACV withdrawal.
Figure 10: HSV-1 latency in L929 cells and reactivation from lysate. (a) There were no plaques in Vero cells overlaid with supernatant from ACV treated HSV-1 infected L929 cells. This indicates that the virus is silenced in the L929 cells. (b) There were plaques in Vero cells overlaid with lysates from ACV treated HSV-1 infected L929 cells. This indicates the presence of viable virus in these L929 cells. (c) % Pixel density of scanned images was used to measure cell survival. % Pixel density normalized against Vero control cells; P= 0.016 by ANOVA.
Figure 11: Maintenance HSV-1 latency in L929 cells and reactivation by TSA treatment. (a) There were no plaques in Vero cells overlaid with supernatant from ACV pretreated HSV-1 infected L929 cells after 48 hours of ACV withdrawal. This indicates that the virus is still silenced in the L929 cells. (b) There were plaques in Vero cells overlaid with supernatants from TSA induced HSV-1 latently infected L929 cells. This indicates the presence of reactivated virus in these L929 cells. (c) % Pixel density of scanned images was used to measure cell survival. % Pixel density normalized against Vero control cells; P=0.003 by ANOVA.
N2A cells cytopathic inhibition assay with ACV treatment

Cytopathic effect assay was conducted to determine the appropriate concentration of ACV protecting N2A cells from cytopathic effect of HSV-1. In these experiments N2A cells start dying when they reach approximately 80% confluency even without treatment and infection. The N2A cells at low confluency about 40-50% were treated with ACV 24 hours before infection and infected with 0.1 MOI. Using high concentrations of ACV (20, 10 µg /ml) caused higher cell death (less cell density) than a low concentration of ACV (5 µg /ml) (Figure 12). The cell survival at low dose of ACV (5 µg /ml) was about 84% compared with N2A control cells and at 20 and 10 µg /ml was 44% and 62%, respectively (Figure 13).
Figure 12: HSV-1 cytopathic effect at different concentrations of ACV treatment of N2A cells. Virus plaques were seen in infected untreated control cells (wells 2). There were no plaques in infected treated cells (wells 8). High ACV concentrations were toxic as noticed by the decrease in the cell density (wells 6 and 7).
Figure 13: HSV-1 cytopathic effect at different concentrations of ACV treatment of N2A cells. Higher concentrations of ACV lead to lower cell survival after HSV-1 infection. Cell survival was determined by measuring % pixel density of scanned images. The pixel density was normalized relative to untreated, uninfected N2A control cells. ACV (5 µg/ml) treated HSV-1 infected cells have higher survival relative to untreated infected control cells P=0.003 by ANOVA.
**HSV-1 latency in N2A cells**

The N2A cells were protected from HSV-1 cytopathic effect by the ACV treatment. The latency of the virus in these cells was confirmed by the absence of plaque formation when the culture media from ACV-treated HSV-1 infected N2A cells were applied to Vero cells (Figure 14, a). Absence of plaques on the Vero cells indicates that the ACV-treated N2A cells were harboring a quiescent virus. Removal of ACV from the culture media did not reactivate the virus. There was no plaque formation on the Vero cells overlaid with the culture media from N2A cells after 48 hours of ACV removal (Figure 15, a). Thus HSV-1 latency was maintained in the N2A cells even after ACV withdrawal.
Figure 14: HSV-1 latency in N2A cells and reactivation from lysate. (a) HSV-1 is silenced in the N2A cells. There were no plaques in Vero cells overlaid with supernatant from ACV-treated HSV-1 infected N2A cells. (b) ACV-treated HSV-1 infected N2A cells have viable virus. There were plaques in Vero cells overlaid with lysates from ACV-treated HSV-1 infected N2A cells. (c) % Pixel density of scanned images was used to measure cell survival. % Pixel density normalized against Vero control cells; P=0.009 by ANOVA.
Figure 15: HSV-1 latency in N2A cells and reactivation by TSA treatment. (a) The virus is silenced in the N2A cells after ACV withdrawal. There were no plaques in Vero cells overlaid with supernatant from ACV pretreated HSV-1 infected N2A cells 48 hours after ACV withdrawal. (b) There were plaques in Vero cells overlaid with supernatants from TSA induced HSV-1 latently infected N2A cells. The plaques indicate the presence of reactivated virus in these N2A cells. (c) % Pixel density of scanned images was used to measure cell survival. % Pixel density normalized against Vero control cells; P< 0.001 by ANOVA.
**HSV-1 reactivation**

Virus was not reactivated in L929 cells treated with ACV after infection. However, L929 cells treated with ACV 24 hours before infection showed virus reactivation in cell lysates and following TSA treatment. Lysates from ACV treated HSV-1 infected L929 cells were titered on Vero cell monolayers. Plaques were seen 2-3 days after lysate application (Figure 10, b). The Vero cells survival decreased by about 85% due to presence of the reactivated infectious virus in the L929 cells lysate (Figure 10, c). Virus reactivation (plaque formation) was observed in 25% of the cultures. L929 cell lysates were prepared by two freeze and thaw cycles at -80 °C. In the TSA reactivation, ACV was removed from the culture media at 48 hours post infection, and then cells incubated for 48 hours without treatment. At 4 days post infection, culture medium was removed and replaced with media containing TSA and cells incubated for an additional 48 hours with TSA in the culture media. At 48 hours from TSA treatment, supernatant fluids from the treated cultures were applied to Vero cell monolayers and examined daily for plaque formation. At the second day post induction, plaques were noticed (Figure 11, b). Vero cells were exposed to supernatant fluid from TSA-treated and infected L929 fibroblasts. These treated Vero cells showed a 50% survival rate compared to control Vero cells (Figure 11, c). The TSA induced HSV-1 reactivation was seen in approximately 31% of the cultures. Both cell lysates and TSA treatment of N2A cells induced virus release from latently infected N2A cells with subsequent plaque formation on Vero cells (Figure 14, b and Figure 15 b). The N2A cells were reactivated in same way as were the L929 cells. In the N2A cell lysates, virus reactivation occurred in 15% of the cultures. Virus released from
the N2A lysates caused a decrease in the Vero cells survival by about 75% (Figure c). Trichostatin A treatment of the N2A cells resulted in virus production from 68% of the cultures. Vero cells survival was decreased by about 80% due to presence of the reactivated virus in the TSA treated N2A cells supernatant fluids (Figure c). Virus induction with TSA treatment was more powerful than in the cell lysates in both L929 and N2A cells.
Quantitative real-time PCR for HSV-1 transcripts

Quantitative polymerase chain reactions (qPCR) were conducted at two time periods (16 hours and 48 hours post infection) in both L929 and N2A cells. In both cell lines, latent infection was established by treating the cells with ACV 24 hours before infection. The following day cells were infected with HSV-1 at an MOI of 1. In the ACV-treated, HSV-1 infected L929 cells at 16 hours post infection, latent infection was confirmed by the decrease in the lytic cycle ICP0 gene transcripts level and increase in LAT gene transcripts level (around one fold) compared with the untreated HSV-1 infected control cells (lytic infection) (Figure 16). At the same time, ICP27 level was elevated in ACV treated HSV-1 infected L929 cells about one and half fold compared with HSV-1 infected control cells. The cause of this elevation might reflect the presence of some cells being lytically infected because latency occurs in only 31% of the cells. At 48 hours post infection, there was a decrease in all gene transcripts, however there was a marked reduction in ICP27 compared with LAT (P=0.03) (Figure 17). At 48 hours post infection, the number of cells was about one fifth of the cells at 16 hours post infection by manual cell counting. In the N2A cells at 16 hours post infection, LAT was observed at low level and ICP0 was decreased in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells. At the same time ICP27 showed approximately one fold increase in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells (Figure 18). At 48 hours post infection there was a reduction in all examined virus genes (Figure 19). The LAT gene was not elevated in the treated N2A cells at either time period. The explanation for this observation might be because the
viral genes were assessed in the whole cell culture and the latency occurred in 68% of cells. Thus, the LAT transcription was masked. Roehm et al., 2011 found that about one third only of cells were LAT positive in cultured vestibular ganglion neurons from ACV-treated HSV-1 infected mice.
Figure 16: HSV-1 latency in ACV treated L929 cells and pattern of viral genes expression 16 hours post infection. RNA was reverse transcribed and the quantity of cDNA synthesized for host and viral transcripts was examined by qPCR. The cDNA copies were determined from triplicate reactions. The cDNA copies were normalized relative to infected untreated L929 cells (virus control). The virus control was set as 1 for each transcript. There was one fold increase in the LAT in ACV treated cells compared with infected untreated cells. However, this result is statistically insignificant; P=0.4 by ANOVA. The increase of ICP27 was not significant statistically; P=0.1 by ANOVA.
Figure 17: HSV-1 latency in ACV treated L929 cells and pattern of viral gene expression 48 hours post infection. RNA was reverse transcribed and the quantity of cDNA synthesized for host and viral transcripts was examined by qPCR. The cDNA copies were determined from triplicate reactions. The cDNA copies were normalized relative to infected untreated L929 cells (virus control). The virus control was set as 1 for each transcript. There was a decrease in all virus genes in ACV treated HSV-1 infected cells compared with infected untreated cells. There was a significant decrease in ICP27 compared with LAT in ACV-treated HSV-1 infected cells; P=0.03 by ANOVA. There was a significant decrease in ICP0 in ACV-treated HSV-1 infected cells compared with infected untreated L929 cells; P<0.001 by ANOVA.
Figure 18: HSV-1 latency in N2A cells and pattern of viral gene expression 16 hours post infection. RNA was reverse transcribed and the quantity of cDNA synthesized for host and viral transcripts was examined by qPCR. The cDNA copies were determined from triplicate reactions. The cDNA copies were normalized relative to infected untreated N2A cells (virus control). The virus control was set as 1 for each transcript. There was an increase in the ICP27 in ACV-treated HSV-1 infected cells compared with infected untreated cells. However, this result is statistically insignificant; P=0.6 by ANOVA. The decrease in the LAT was not significant statistically; P=0.1 by ANOVA.
Figure 19: HSV-1 latency in N2A cells and pattern of viral gene expression 48 hours post infection. RNA was reverse transcribed and the quantity of cDNA synthesized for host and viral transcripts was examined by qPCR. The cDNA copies were determined from triplicate reactions. The cDNA copies were normalized relative to infected untreated L929 cells (virus control). The virus control was set as 1 for each transcript. There was a decrease in all viral genes of ACV treated HSV-1 infected N2A cells compared with the control; P<0.001 by ANOVA.
Table 1

Quantitative Real-Time PCR primers and probes (Danaher et al., 2005)

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<th>Sequence</th>
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DISCUSSION

After primary infection, herpes simplex viruses have the ability to remain latent in the neuronal cells with occasional reactivation and shedding during subsequent lytic infection. To date, the mechanism for establishment of a quiescent infection and reactivation is not well understood. In this study, L929 cells and N2A cells were used as models for HSV-1 latency establishment and reactivation in vitro. L929 cells were chosen as a model because Scheck et al., 1986 have shown that HSV-1 latency can be established in human fetus lung fibroblast (HFL-F) cells by treating them with ACV and interferon-α (IFN-α) 24 hours before infection. HSV-1 replication was suppressed in L929 cells treated with IFN-γ (Frey et al., 2009). Normal human diploid fibroblasts (NHDFs) showed wild type HSV-1 quiescent infection when they were serum starved. Latency was confirmed by accumulation of LATs in these cells (McMahon and Walsh, 2008). Neuro-2A cells are mouse neural crest-derived cell lines. N2A cells were chosen because they are neuronal phenotype.

Acyclovir (ACV) has been useful in maintaining HSV-1 latent in many studies in vivo and in vitro. Miller et al., 2003 showed that ACV can induce HSV-1 latency in QIF-PC12 cells and in rabbit eye because ACV does not interfere with viral entry and subsequent transmission to the nucleus of PC12 cells. Cytopathic inhibition assays were used to determine the appropriate concentration of ACV for suppression of HSV-1 replication with low cytoxicity to the cell lines. In the L929 cells in this study, high concentrations of ACV (40 μg /ml and 20 μg /ml of media) were toxic. However, a low concentration of ACV (10 μg /ml of medium) protected L929 cells from the cytopathic
effect of HSV-1; no plaques formation resulted, with 80% cell survival in ACV treated HSV-1 infected cells compared with HSV-1 infected untreated cells. HSV-1 established latency in L929 cells treated with ACV 24 hours before infection. Subsequent virus reactivation occurred when ACV-treated, HSV-1 infected L929 cells were treated with TSA and cell lysates. On the other hand, treatment of L929 cells with ACV after infection was less toxic to the cells than treatment before infection; a suppressed virus replication and a quiescent state apparently did not result.

In N2A cells, low concentration of ACV (5 µg/ml of medium) prevented the cytopathic effect of HSV-1 when the cells were treated 24 hours before infection. This concentration was not toxic to the N2A cells with 84% cell survival in comparison with higher concentrations of ACV (10 and 20 µg/ml of medium). Establishment of latency in both L929 cells and N2A cells was confirmed by absence of virus progeny in the culture medium when examined for plaque forming units (PFU).

HSV-1 latency in L929 cells and N2A cells was maintained even after removal of ACV from culture media. Maintenance of latency in ACV treated HSV-1 infected cells after ACV withdrawal was confirmed by absence of plaques when the supernatant fluids from these cultures were titrated on Vero cells monolayer. This observation is consistent with the findings of others (Danaher et al., 2005; Miller et al., 2003; Scheck et al., 1986; Danaher et al., 1999a; Danaher et al., 1999b). In contrast, other studies have shown that removal of ACV from culture medium of rabbit corneal cells (SIRC) (Rong et al., 1988) and cultured vestibular ganglion neurons (VGNs) (Roehm et al., 2011) induced viral reactivation from latency. There is no clear explanation for this difference. L929 and N2A cell lysates induced decreases in Vero cells survival due to the cytopathic effect of the
released HSV-1 virus from ACV-treated latently infected cells.

Histone acetylation has an impact on HSV-1 reactivation. HSV-1 DNA associated with deacetylated histones in human fetal lung fibroblasts (MRC5) quiescently infected with replication-defective HSV-1 mutants (Coleman et al., 2008). De-repression of the viral genome by HSV-2 superinfection or ICP0 delivery by adenovirus vector revealed histone hyperacetylation of HSV-1 genome. The hyperacetylation of histone 3 (H3) occurred more at LAT promoter and enhancer than at the other DNA regions following genome de-repression (Coleman et al., 2008). Trichostatin A has been used successfully to induce HSV-1 reactivation from quiescently infected PC12 cells (Danaher et al., 2005). Quantitative real-time polymerase chain reaction revealed that latency-associated transcript (LAT) is the only transcript elevated in response to TSA treatment of PC12 (Danaher et al., 2005). The wild type HSV-1 and LAT mutant HSV-1 were equally reactivated in response to TSA treatment. Thus, researchers suggested that LAT does not exert a direct role on reactivation from latency. However, Devi-Rao et al., 1994 revealed that murine trigeminal and lumbosacral ganglia infected with LAT- mutant showed delayed reactivation in comparison with ganglia infected with wild type or LAT+ rescuant HSV-1. In our study, TSA application to L929 cells and N2A cells for 48 hours caused virus release and reactivation from latency. Induction of reactivation from latency was observed by plaque formation when supernatant fluid from TSA treated cultures was added to Vero cells monolayer.

As expected, our finding of quantitative real-time PCR (qPCR) revealed an increase in LAT expression in ACV treated HSV-1 infected L929 cells 16 hours post infection as compared with untreated infected control cells. At same time, ICP0 was
decreased, but ICP27 was elevated. A possible explanation for the elevation of lytic gene transcript (ICP27) might be that some cells in the culture were undergoing lytic HSV-1 infection. At 48 hours, all viral genes (LAT, ICP0, and ICP27) were suppressed. The decrease of LAT level might because of dying the cells that were latently infected with HSV-1. The cells at 48 hours post infection were only one fifth of the cells at 16 hours post infection. Previous study in our lab (data not published) showed that only 20-30% of FUDR + IFN-γ treated and quiescently HSV-1 infected L929 cells were survive by HOECHST staining at 12 hours post infection. In the N2A cells LAT was observed at low level and ICP0 was decreased in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells at 16 hours post infection. At the same time ICP27 showed approximately one fold increase in ACV-treated HSV-1 infected N2A cells as compared with the untreated HSV-1 infected N2A cells. At 48 hours post infection, LAT, ICP0, and ICP27 were decreased in ACV treated HSV-1 infected cells as compared with the control untreated infected cells. The LAT gene was examined in the whole cell culture instead of a single cell level. That might mask the LAT gene expression and explain why LAT was not high in the ACV-treated HSV-1 infected N2A cells. In vitro, about one third only of cells were LAT positive in cultured vestibular ganglion neurons after ACV treatment (Roehm et al., 2011). Furthermore, the HSV-1 latency might happen in the absence of LAT gene. LAT is not a mandatory for HSV-1 latency establishment (Javier et al., 1988; Block et al., 1990). Southern blot analysis of virus gene transcripts produced during the establishment and maintenance of latency in quiescently HSV-1 infected PC12 cells revealed the presence of lytic α gene transcripts. The α gene transcripts were maintained as long as 10 days after ACV withdrawal. At the same time,
the LAT was consistently expressed (Figure 20) (Danaher et al., 1999a). The qPCR results obtained in the present study at 16 hours post-infection are consistent with those of Danaher et al., 19991. While they observed ICP0, ICP4 and ICP27 transcripts along with LAT at 5-10 days post infection, LAT transcripts alone was not seen until 20 days post infection.

![Figure 20: Southern blot analysis of HSV-1 gene transcripts expressed during establishment and maintenance of quiescent infection in PC12 cells (Adapted from Dana-her et al., 1999a)](image)

In the future, using green fluorescent protein (GFP) to tag HSV-1 DNA would provide better visualization of HSV-1 positive cells by microscopic examination. Roehm et al., 2011 used TSA to induce HSV-1 reactivation in cultured vestibular ganglion neurons (VGNs) which are HSV-1 GFP positive after ACV removal. After ACV removal, the remaining cells were HSV-1 GFP positive. Another advantage for using HSV-1 GFP
virus is that HSV-1 can be used at a very low multiplicity of infection (MOI). Kuhn et al., 2013 infected geniculate ganglion neurons (GGN) with HSV-1 GFP at an MOI of 0.004 in presence of ACV. In our present study, we noticed elevated LAT only in ACV treated HSV-1 infected L929 cells at 16 hours post infection at same time the ICP27 was elevated suggesting that either virus replication had started and not yet been stopped and/or might be because of some of the cells in the cultures did not contain latent virus. In our study virus was reactivated from quiescence in 31% of TSA-treated fibroblast cultures and in 68% of TSA-treated N2A cultures. If cells containing latent virus can be sorted using HSV-1 GFP-tagged DNA and those sorted cells submitted to qPCR analyses greater percentages of cultures treated with TSA should yield reactivated HSV-1.

Previous studies in our lab (data not published) used anti-mitotic agent 5-fluoro 2’deoxy uridine (FUDR) and IFN-γ to suppress HSV-1 replication and induce a quiescent state in the L929 cells. The virus was silenced in these cells. However, LAT gene was not detected in treated HSV-1 infected L929 cells at 16 hours post infection by RT-PCR analysis. We can mimic the neurons, which are in post mitotic state by treating the L929 cells with FUDR and inhibit the HSV-1 replication with ACV treatment to induce HSV-1 latency in L929 cells in the future studies.

Differentiation of N2A cells can be accomplished by serum deprivation or treatment with forskolin (cAMP inducer), retinoic acid (RA) (vitamin A derivative), and 2, 4-dinitrophenol (agent has been known to protect cells from neurotoxicity) (Tremblay et al., 2010). Neuronal differentiation methods of N2A cells do not provide information about the neuronal types. Differentiation of N2A cells to mimic sympathetic neurons might provide better HSV-1 latency.
REFERENCES


