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HSV-1 Infection of C3H Central Nervous System Cell Lines

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HSV-1 Infection of C3H Central Nervous System Cell Lines

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Lauren Kay Van Buren ENTITLED HSV-1 Infection of C3H Central Nervous System Cell Lines BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Herpes Simplex Virus Type 1 (HSV-1) can infect the nervous system, resulting in a disease known as herpes encephalitis (HSE). Herpes encephalitis affects thousands of people each year; many cases are fatal or permanently debilitating. Approximately two thousand known cases occur in the United States each year alone (Neuroland online source). Acyclovir has been the drug of choice used to treat herpes encephalitis. Even after the administration of acyclovir, permanent neurological damage and/or death often results. Thousands of individuals would benefit by the discovery of drugs that are more effective at preventing lasting HSE damage and death. Knowledge concerning HSE infection of the brain could be advanced with the development of co-culture systems that allow for the study of one specific cell type or a subpopulation of cells during an active infection.

Additional models are needed to test therapies against HSE. The majority of models that currently exist are *in-vivo* or primary cell line models. Even though many of these models can be used to mimic what actually occurs during herpes encephalitis infection in humans, these systems have several weaknesses. The usage of *in-vivo* models requires a great deal of time, preparation, funding, and care by a veterinarian staff. Primary cell lines can be difficult to isolate and maintain for long lengths of time in cell culture.

Unlike primary cell lines, continuous cell lines are able to be used for longer periods of time. Many can be easily purchased from cell banks such as the American

Type Culture Collection (ATCC) for a reasonable price. Continuous cell lines are easy to culture and can be used in many different types of experiments. The use of a continuous cell lines prevents animal suffering and death.

The purpose of this thesis was to determine if cell lines from a C3H murine strain can accurately mimic viral production that occurs during an actual HSV-1 infection in the central nervous system. One cell line is a pure microglial cell line. Three other cell lines, derived from P19 cells, were also tested. One cell culture tested was a system composed of mixed brain cells such as neurons, astrocyte-like cells, and fibroblast-like cells. Another culture evaluated contained only neuronal cells. P19 cells were evaluated as well. The results indicate that only the microglial and mixed cell cultures supported viral production that mirrors what occurs in animal models. The neuronal cells not only failed to produce virus, but the neurons survived for less than twenty-four hours in culture after in infection with HSV-1.

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Dedication

I dedicate this thesis in loving memory to my mother, Mary Ann. Everything noteworthy and positive that I have accomplished or will ever accomplish is a direct reflection on her. She was an exceptional mother and an outstanding mentor. Few people have ever been faced with the challenges, disadvantages, and bad luck that plagued Mary Ann throughout her life. However, she accomplished a great deal and earned a great deal of respect in spite of everything that she was dealt. I will always love her.

Background Literature

HSV-1. Herpes Simplex Virus Type 1 (HSV-1) has an icosahedral nucleocapsid that is surrounded by an envelope covered with many tiny spikes that contain important viral glycoproteins (Voyles 2002). In order for the brain to develop herpes simplex encephalitis (HSE), HSV-1 must first infect numerous cell types and successfully travel from the initial site of infection to the central nervous system (CNS). HSV-1 must first infect epithelial cells that are present on the surface of the skin, the mouth, or the eye (Wang 2005). During attachment, the viral glycoprotein spikes first attach to the extracellular matrix surrounding the target epithelial cell. Then, gB and gC (both spike glycoproteins) proceed to bind to the heparan sulfate on target epithelial cells. Heparan sulfate is a large polysaccharide that extends out from the protein core of the epithelial cell. After this virus has made contact with the cell through its heparan sulfate, other glycoproteins of the virus then proceed to attach to the cell. Next, gD binds to a herpes entry mediator receptor. This initiates the process of penetration (Voyles 2002). After the epithelial cells have been infected, the virus can infect other epithelial cells as well as nearby neuronal cells.

Neuronal Cells. Neurons are cells of the nervous system that have the ability to carry electrical and chemical forms of communication from one part of the body to another part. A neuron is composed of a cell body and processes that extend out from the cell body known as neurites. Neurites can be either axons or dendrites. Axons are responsible for carrying action potentials from the body of a neuron to a target. At the

end of the axon, there is a swelling present (known as the presynaptic terminal) where neurotransmitters are released to target cells (Purves 2004). Neurotransmitters are chemical signals that are sent among neuronal cells. Dendrites project out of the receiving neural cell bodies; dendritic postsynaptic terminals receive synaptic impulses or neurotransmitters from the axons of neighboring cells (Purves 2004). Growth cones are "specialized structures" at the tip of axons that are responsible for exploring the external environment and guiding growth in a particular direction (Purves 2004). Varicosities are identified as irregular shaped bulges in the axon that can be up to 8 times the diameter of the axon. Varicosities are usually found at axon branch points (Saksena 2006**).**

HSV-1 Infection of Peripheral Nerve Tissue. This virus initially infects surface epithelial cells that are part of a mucosal membrane, like the mouth. After infecting the surface epithelial cells, HSV-1 spreads to the axon fibers of peripheral nerves that are responsible for innervating the surrounding tissue. Recent published data suggests that HSV-1 virions must bind to a series of receptors in order to attach to and fully penetrate neurons. It is believed that HSV-1 glycoproteins gC and/or gB must bind heparan sulfate; gD is believed to bind nectin-1 (Immergluck 1998; Richart 2003). However, other receptors, necessary for viral attachment and penetration, may also exist.

In the synapse region, neurons have two main junctions with distinct functions. The junctions are known as synaptic and puncta adherentia junctions. Both junction types are potential sites for viral penetration. Puncta adherentia junctions are adhesion sites between neural axons and their targets. Puncta adherentia junctions are formed by adhesion molecules known as nectins. Some nectins are receptors for HSV-1 gD. There are four types of nectins: nectin-1, nectin-2, nectin-3, and nectin-4. The extracellular region on a nectin molecule has three Ig-like loops. This region is followed by one

transmembrane region and then a cytoplasmic region. The cytoplasmic region binds to afadin. Afadin is a binding protein that connects the nectins to the actin cytoskeleton of the cell (Takai 2003). Nectin-1 extends out from the presynaptic side, and Nectin-3 extends out from the postsynaptic side at a synapse. Nectin-1 and Nectin-3 form a hetero-trans-dimer to make the adhesion complete (Takai 2003). Studies have shown that nectin-1 is the primary receptor that HSV-1 gD binds to when infecting neurons (Richart 2003). However, additional HSV-1 binding receptors on puncta adherentia or synaptic junctions cannot be ruled out at this time. Some clinical strains of HSV-1, responsible for causing herpes encephalitis, have gD molecules that are capable of binding to nectin-2 (Krummenacher 2004).

In one study, transmission electron microscopy was used to study in-vitro HSV-1 infection in neurons using human fetal dorsal root ganglion. The dorsal root ganglion is a component of the PNS. Special attention was paid to the following processes: attachment, penetration, and release. In order to study attachment and penetration, the neurons were first infected with HSV-1 virus. Then, these neurons were fixed and processed for electron microscopy at 90 minutes post infection. Enveloped viral capsids were attached to the outside of the axon. Unenveloped virions were seen in the interior of the axon. However, partially enveloped capsids were not present, nor were enveloped capsids present inside the axon (Saksena 2006). The absence of enveloped capsids inside the axon support entry by fusion, rather than entry by endocytosis. Endocytosis would most likely result in the presence of enveloped capsids inside the axon (Saksena 2006).

After the virus has successful penetrated the neuron, the virus then makes its way to the neuronal cell body where the viral DNA can reside latent in the nucleus. The virus can later replicate, leave the cell body, and travel to the axon of the neuron. In one

experiment, infected neurons were observed with an electron microscope. This study determined that it took between 19 and 24 hours after infection for *de novo* synthesized virus to enter the axon. The exact nature and location of viral release has not been fully determined at this time. Some investigators have noted the presence of enveloped HSV-1 capsids in the proximal area of the axon near the cell body. Some have hypothesized that this could be a potential area where the virus is released.

New evidence is emerging that may indicate other potential sites of viral release. Strong evidence that viral release occurs at axon terminals has recently been published by one group of researchers. Unenveloped viral capsids have been observed in the middle of axons. Unenveloped HSV-1 capsids were also observed in areas between the growth cones and varicosities. Enveloped, unenveloped, and partially enveloped virions were identified in growth cones and varicosities of neurons (Saksena 2006). Confocal microscopy was used to indicate where VP5 (a capsid protein), UL37 (a tegument protein), and gG (an envelope glycoprotein) were expressed in the axon. Although staining for all three was present throughout the entire axon, colocalization of all three proteins was mainly detected in the varicosities and growth cones of terminal axons. Thus, the terminal regions of axons may be likely sites for viral release (Saksena 2006).

The area of the cell body and the areas of proximal axons cannot be excluded as sites for viral release since enveloped capsids have been located in these regions as well. These factors have led some scientists to the conclusion that there are potentially two main sites where viral egress occurs. One theory is that enveloped HSV-1 capsids might be released from areas of the cell body or proximal axon. There may also exist a mechanism in the axon that only allows unenveloped capsids entry into mid and distal

axons. These unenveloped capsids travel to the growth cones and varicosities were they are enveloped and released (Saksena 2006).

HSV-1 Infection of the CNS. Once the virus travels to the brain through peripheral neural pathways, HSV-1 is able to infect not only neurons, but also microglial cells, astrocytes, and oligodendrocytes (Immergluck 1998). Microglial cells, astrocytes, and oligodendrocytes are three types of neuroglial cells that are present in the CNS. Astrocytes play a key role in maintaining a proper chemical environment in order to allow neuronal signaling. Oligodendrocytes are responsible for myelinating the axons of neurons. Microglial cells are considered to be macrophage cells by many scientists; however, some scientific communities still consider them to be distinct from macrophage cells. They are the "scavengers" of the CNS; they remove unwanted debris. During infection, these cells are able to secrete signaling molecules like cytokines (Purves 2004). In one study, many of these different cell types showed variable immunoreactivity to nectin-1 (Guzman 2006). At this time, it is not clear if nectin-1 is the primary receptor used by HSV-1 to infect these cells.

Vahlne et al. used cell fractions harvested from brains to study the amount of virus that was absorbed upon infection. The cell fractions were prepared from the following CNS cell types: neurons, astrocytes, and synaptosomes (synaptic regions of neurons). Cells from rats, rabbits, and monkeys were first tested. HSV-1 was absorbed by astrocytes and synaptosomes. Synaptosomes absorbed a greater percentage of virus than did astrocytes. The neural cell bodies were largely unsuccessful at absorbing virus. HSV-1 was more readily absorbed than HSV-2. A similar experiment was conducted using the same types of cells from mice. Analogous results were found, except that murine astrocytes absorbed similar amounts of HSV-1 and HSV-2. Using human brain

tissue, both synaptosomes and astrocytes absorbed HSV-1 virus. However, synaptosomes absorbed more virus than astrocytes. Neural cell bodies were unsuccessful at absorbing a majority of the virus. Even though the human cells were able to absorb HSV-1, very little HSV-2 was absorbed. The results from human sources were similar to the results obtained with the rat, rabbit, and monkey cells (Vahlne 1980).

Immune Response to HSV-1 in the CNS. Once infection in the CNS occurs, microglia and astrocytes are believed to form an active immune response with cells that migrate into the CNS during times of infection. The cells that migrate into the CNS include: lymphocytes, monocytes, and neutrophils. CD8+ T-lymphocytes act to decrease virulence and spread of the virus through nonlytic methods, involving cytokines. Microglia are producers of chemokines that attract the lymphocytes to sites of infection in the CNS. Microglia are believed to be the primary producers of cytokines in the CNS (Lokensgard 2001).

During an active infection, CNS cells also differ in the quantity of virus produced by each cell type. When infected, microglia initially produce only a limited amount of virus. The production of virus rapidly declines in these cells as time passes. Astrocytes and neurons are the main producers of virus during an active infection (Lokensgard 2001). A cell culture of human oligodendrocytes, infected with HSV-1, produced a large amount of virus; virus production increased with time (Bello-Morales 2005).

Clinical Aspects of HSV-1 Infection in the CNS. HSV-1 is capable of causing serious and lethal damage to the central nervous system. HSE is one of the main causes of acute sporadic encephalitis in both adults and children (Tyler 2004). Neonatal HSV-1 infection can often damage infant brain development. Burgos et al. noted that the majority of

neonatal herpes infections are caused by HSV-2. However, thirty percent are caused by HSV-1 (Burgos 2006).

HSV-1 infections in the CNS have caused brainstem encephalitis, myelitis, and localized or diffuse HSE infections throughout the brain (Bovin 2004). Patients can be grouped into having localized or diffuse HSE. Localized HSE is more common than diffuse HSE. Localized disease causes weakness, problems with the sensory system, aphasia, visual field defects, cranial nerve palsies, and/or abnormalities present on neuroimaging studies. Diffuse disease causes patients to experience changes in personality and/or decreases in levels of consciousness. Abnormalities, caused by diffuse HSE, are not detected with neuroimaging equipment. Therefore, this form of infection can be harder to recognize (Tyler 2004).

Polymerase chain reaction (PCR) of cerebral spinal fluid (CSF) is the primary diagnostic tool used for detecting the presence of HSV-1 in the CNS. Magnetic Resonance Imaging (MRI) is used to locate apparent abnormalities in the brain during a HSE infection. When localized HSE is present, MRIs often find edema and necrosis occurring in the temporal lobe region (Tyler 2004). Upon diagnosis of HSE or when HSE is suspected, patients receive intravenous acyclovir (10 mg every 8 hrs for 14-21 days). The use of this drug has reduced mortality from approximately 70% to 28%. In most cases, this drug causes a severe decline in the amount of PCR-positive cases within two weeks. The treatment outcome is influenced by the following variables: age, level of consciousness when treatment was started, duration of the HSE infection, and viral load at the time treatment was started. Unfortunately, relapses can occur after acyclovir therapy. When relapse occurs, PCR is again performed on CSF specimens from the patient. If no virus is detected in the brain, relapse could be caused by a non-viral

immune-mediated encephalomyelitis which can cause demyelination of the neurons. If virus is detected in CSF, then relapse is most likely from an active HSE infection (Tyler 2004).

Individuals who do recover are often severely impaired even if appropriate antivirals are administered (Tyler 2004). Since harmful viral production is a critical component of pathogenesis resulting from an active infection, it logically follows that preventing widespread viral production would reduce the severity of the symptoms caused by an active HSV-1 infection. Acyclovir is currently the drug of choice that is used to combat HSV-1 infection in the CNS. However with the emergence of drug resistance in viruses, more anti-HSV drugs and therapies need to be developed. Better drugs need to be developed that reduce viral load more significantly than acyclovir and that prevent serious sequel from occurring. It would also be beneficial to have models that allow that effects of active infection to be studied using one specific cell type or a specific subpopulation of cells.

Models used to study HSV-1 Infection. Several models have been developed to study HSV-1 infection. The majority of models are *in-vivo* or primary cell line models. Even though many of these models can be used to mimic what actually occurs during herpes encephalitis infection in humans, these systems have weaknesses. The usage of *in-vivo* models requires a great deal of time, preparation, funding, and care by a veterinary staff. When an animal is infected with HSV-1, the animal may experience pain and suffering. An infected animal is killed by the termination of the experiment. Difficulties may also emerge when trying to use primary cell lines to study the effects of an active HSV-1 infection. A great deal of work is generally required to isolate specific cells from an animal. The establishment of a primary cell line can be unsuccessful due to

contamination from other cell types or harmful microbes. Many primary cell lines can only be maintained for a short period of time. After a primary cell line is no longer viable, additional animals are used to provide additional cells.

Unlike primary cell lines, continuous cell lines are able to be used for longer periods of time. Many can be easily purchased from cell banks such as the American Type Culture Collection (ATCC) for a reasonable price. Continuous cells lines are easy to culture and can be used in many different types of experiments. They can be easily stored and used numerous times. Continuous cell lines are not associated with the costs involved with maintaining and caring for animals used in *in-vivo* experiments or the continuous harvesting of primary cells.

C3H murine continuous cell lines are available that are either cells from the central nervous system or cells that can be induced to differentiate into cells of the central nervous system. The purpose of this research is to determine if several CNS cell cultures derived from C3H mice can be effective models to study HSV-1 production during an active infection. Previous research has used both *in-vivo* models and *in-vitro* models to study viral production during an active HSV-1 infection. In this thesis, C3H cell lines are evaluated based on viral production. Both *in-vivo* and *in-vitro* experiments have demonstrated that neurons and astrocytes are capable of producing virus. Nonproductive infection is usually apparent in HSV-1 infected microglia.

Lokensgard and colleagues conducted experiments on primary cell cultures from human fetuses. The three cell cultures that were tested were: a cell culture comprised of neurons and 10% neuroglia, one of astrocytes, and one of microglial cells. HSV-1 virus was produced by the cultures composed primarily of neurons and astrocytes. Microglial cells produced only a small amount of virus. The majority of the infection was

nonproductive (Lokensgard 2001). Completely formed virus has been located in the astrocytes and neurons of autopsied humans and animals who died due to herpes encephalitis infections (Landolfi 2005; Yamada 2003; Weissenbock 1997).

Frye infected C3H mice with $1x10^6$ pfu/eye of HSV-1, strain 17+. The virus spread to the brain. The brain was aseptically removed from mice at 2, 4, 6, and 10 days post infection. The brains were then homogenized and afterwards the remaining cellular debris was removed through centrifugation. Plaque assays were then performed on the supernatant fluids in order to determine viral titer. Viral titer started to increase around Day 6 post infection. On day 10 post infection, the viral titer reached the maximum level (23 pfu/ml) (Frye 2001).

One purpose of these experiments was to create a HSV-1 cell culture system that can be used as an effective model to test anti-virals that are designed to decrease viral production. Another purpose of these experiments was to create models of subpopulations of CNS cells that could be studied during active infection. These models must mimic what actually occurs *in-vivo*. These models must respond to anti-virals the same way that these cell types would *in-vivo*. For example, acyclovir, the drug of choice for treating herpes encephalitis, decreases viral production in both *in-vivo* and *in-vitro* models. In order for these cell culture models to be effective, acyclovir should also decrease viral production in these cell cultures.

Hypothesis. The hypothesis of this study is that CNS cell lines from the C3H mouse can be used as models to study HSV-1 viral production by different cell types, namely undifferentiated P19 cells, neurons, microglial cells, and a mixed cell population containing neurons, astrocyte-like cells, and fibroblast-like cells during a an active HSV-1 infection.

Materials and Methods

Over twenty years ago, McBurney (Jones-Villeneuve 1982) developed the P19 cell line (ATCC CRL-1825). This cell line is derived from a C3H murine embryonal teratocarcinoma cell. P19 cells are stem cells that can differentiate into a broad range of other cell types after exposure to certain chemicals. Upon exposure to dimethyl sulphoxide (DMSO), the P19 cells rapidly differentiate into cardiac and skeletal muscle cells. When these same cells are exposed to retinoic acid, P19 cells rapidly develop into neuronal and neuroglial cells of the brain. In previous research, P19 cells were exposed to retinoic acid and some of the subpopulations stained positive to either Neurofilament 160 or GFAP. Cells with neuronal morphology stained positive for Neurofilament 160 (used to indicate the presence of neurons). Many of the cells stained positive for Glial Fibrillar Acidic Protein (GFAP). Astrocytes are the only known cells that express GFAP (Jones-Villeneuve 1982).

The overall intent of this experiment was to quantify viral production within the first 48 hours after infection. Consequently, my experiment included separate analyses of undifferentiated P19 cells, mixed brain cells, microglial, and neuronal cells. In this study, C3H embryonal teratocarcinoma cells (P19) were exposed to retinoic acid. Retinoic acid changed the P19 cells into a mixed population of brain cells (Jones-Villeneuve 1982). From this cell culture mixture, neuronal cells (post mitotic cells) were isolated by using cytosine arabinoside (a DNA synthesis inhibitor). Therefore, P19, mixed brain cells, and

isolated neuronal cells comprise three of the four cell cultures studied. A different C3H microglial cell line (ATCC CRL-2467) was also studied**.**

Determining viral production in the four cell lines involved several stages. The stages involved infecting different CNS cell types with HSV-1, harvesting the supernatant fluids surrounding the cells at specific time points, and analyzing the virus titer. The next stage of the experiment was plaque assay. The purpose of this part of the experiment was to quantitate the amount of virus produced by the various cell types at specific times after infection. Another experiment was conducted to determine if the cells of the mixed brain cell culture were capable of *de novo* synthesis.

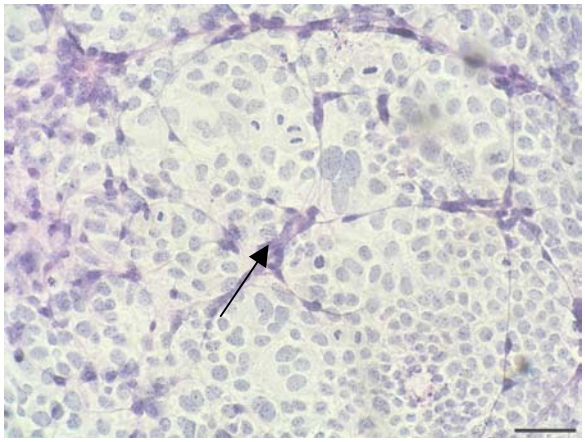

Figure 1.Bundles of neuronal cell bodies lie upon astrocyte-like cells. The arrow points to a bundle of neuronal cells with processes extending out from the cell bodies. The measurement bar represents 50µm.

Preparation of cells prior to infection. The different cell types were placed in the wells of twelve well plates prior to being infected with 1 MOI of virus. The cells were suspended in the media noted in *Table 1* and *Table 2*. When testing the microglial cells and the P19 cells, $2.5x10^5$ cells were placed in each well and incubated overnight before being infected with 1 Multiplicity of Infection (MOI). MOI is defined as infectious viral particles over the number of cells prior to infection.

In order to prepare the mixed brain cells, P19 cells were first incubated in 100 x 15 mm petri dishes containing media with $5x10^{-7}$M of retinoic acid for a period of four days. The media was replenished every two days. After the four day incubation period, the cell aggregates were removed and resuspended in fresh media. A cell count was performed, and $5x10^5$ cells were added to each well of the 12 well plate. The media was changed every 2 to 3 days as needed. After six days had elapsed, a cell count was taken of four wells using a hemocytometer. The average cell count of the four wells was then determined. This number was taken as the approximate number of cells in each well of the plate. Then, 1 MOI of virus was added to three wells.

In order to prepare the isolated neuronal cells, P19 cells were first incubated in petri dishes containing media with $5x10^{-7}$M of retinoic acid for a period of four days. The cells were given fresh media every two days. After four days, the cell aggregates were collected. A cell count was performed using a hemocytometer, and $1x10^6$ cells were added to each well of the 12 well plate. After a cell count had been taken of the cells in media, $1x10^6$ cells were placed into each well of a laminin coated 12-well plate. Each well contained at least 1m of media. The cells were then incubated overnight. The next day, old media was removed, and Neuralbasal media containing B-27 supplement, 5µg/ml of cytosine arabinoside (ArcC), and 0.5M L-glutamine were added. There was at least 1ml of media in each well. The media was changed every 2 to 4 days and replaced with fresh media. Total incubation time with the cytosine arabinoside was approximately 6 days. After six days, the cells were switched to regular Neuralbasal media with just the B-27 supplement and L-glutamine. The cells were exposed to the new media overnight before being infected with virus. The next morning, a cell count was taken of four wells. This average number of all four cell counts was taken to be the approximate value of the

number of cells present in each well. Then, three of the wells were infected with 1 MOI of virus.

Table 1. Media used for culturing the P19 Cells

Cell Types	Undifferentiated P19 Cells and Differentiated P19 cells	P19 cells being induced with retinoic acid	Mixed brain cell culture plating stage immediately after induction	Mixed brain cell culture during the neuronal isolation stage	Isolated neurons exposed to HSV-1
Media Used*	α-Minimal Essential Media (α-MEM), 10% Calf Serum (CS)	α-MEM, 5% CS	α-MEM, 10% CS	Invitrogen Neuralbasal media, Invitrogen B-27 supplement, .5mM of L-glutamine, cytosine arabinoside (5µg/ml)	Invitrogen Neuralbasal media, Invitrogen B-27 supplement, .5mM of L-glutamine

Table 2. Media used for culturing Vero and Microglial Cells

Cell Type	Vero Cells	Microglial Cells
Media Used*	Dulbecco's Modified Essential Media (DMEM), 10% CS	DMEM, 10%CS, 20% LADMAC conditioned media

*Based on previous research, all derivatives of P19 cells were exposed to 1xGentamycin Sulfate solution. Microglial cells and Vero cells were exposed to 1xPen-Strep Solution. All cells were incubated in an incubator at 37ºC, 5% CO_2.

Table 3. Procedures for Preparing Isolated Neuronal Cells

Procedures	Incubate P19 cells with retinoic acid	Incubate differentiated cells with ArcC and serum-free Neuralbasal media for 6 days	Infect the cells with 1 MOI of HSV-1 virus
Purpose	Allows the cells to differentiate into a mixed population of neurons, astrocytes, and fibroblast-like cells	ArcC and serum-free media will kill all of the cells, except for the neurons. This procedure allows for the isolation of the neurons.	The virus will be allowed to infect the cells.

Infectivity Assays. Each experiment was performed in triplicate. Each well was infected with approximately 1 MOI of HSV-1, 17+ strain. Supernatant fluids from three wells were harvested at the following time points post infection: 6 hrs, 12 hrs, 24 hrs, 36 hrs, and 48 hrs. After supernatant fluids had been collected from each well, 2 mls of fresh media were added back to each of the wells. After the supernatant fluids were collected, they were centrifuged and filtered with .45µm sterile filters to remove any extraneous cell debris. The filters allowed virus to pass through. All supernatant fluid samples were titered using a plaque assay.

Plaque Assay Protocol. In each well of a 12-well plate, 1.25 million Vero cells were placed and allowed to attach overnight. The Vero cells were suspended in MEM with 5% CS. The entire plate was incubated until confluent monolayers formed in each of the wells. Then, ten fold dilutions were made of the supernatant fluids from the infected cells. Each well was rinsed with serum-free MEM. The dilutions were added to separate wells in the 12-well plates. In each well, 250µl of virus infected media from each dilution was placed in each well of the 12 well plate. The plate included two wells that did not contain any virus. Those wells served as controls. The plate was then incubated for one hour. During this time, the plate was occasionally shaken. After incubation, the supernatant fluid dilutions were removed from the different wells. Then, 2 mls of 2% methyl cellulose were added to each well. The plate was incubated for 4-5 days. Then 1 ml of formalin solution was added to each well. This solution was left in the wells for 30 minutes to fix the cells and kill virus. After the appropriate incubation period, the formalin solution was aspirated, and the wells were gently rinsed with tap water if large amounts of methylcellulose remained. Next, 1 ml of crystal violet dye was added to each well for 30 minutes. Then, the crystal violet was aspirated out of the wells. The wells

were rinsed with tap water. The plate was turned upside down on a paper towel in the virus hood and left for 1-2 days until the bottoms of the wells were sufficiently dry. Next, visible distinguishable plaques were counted. Then plaque forming units (PFUs) were determined for each well using the formula below. All the PFUs for each counted well were averaged together to generate the overall PFU number assigned to each plate.

Table 4. Illustration of a Plaque Assay Setup

No virus/control	No virus/control	10^{-4} dilution	10^{-4} dilution
10^{-3} dilution	10^{-3} dilution	10^{-2} dilution	10^{-2} dilution
10^{-1} dilution	10^{-1} dilution	no dilution	no dilution

The formula used to determine pfu from plaque assays is *(plaque number x reciprocal dilution x reciprocal volume in ml)*. An example of this would be if 40 plaques were counted from one well infected with a .25 ml of a 10^{-5} virus dilution, the PFU would be determined $40x(10^5)x4=1.6x10^8$ pfu/ml.

Acyclovir Experiment. The purpose of this experiment was to determine if *de novo* synthesis was occurring in the mixed brain cells. The addition of acyclovir to many types of cells infected with HSV prevents *de novo synthesis*. If titers from wells with acyclovir and wells without acyclovir were similar, then that would have indicated that *de novo* synthesis was not taking place. A difference in viral titer between wells with acyclovir and wells without acyclovir would indicate that *de novo* synthesis was occurring.

P19 cells were first differentiated with retinoic acid as described in the previous experiment above. Then $5x10^5$ cells were placed into each well of a 12-well plate and allowed to mature for a period of 6 days. After the six day period of incubation, the cell count from four of the wells was determined as explained in the previous section. The average of the four wells was taken as the average cell count in each well. Three wells were incubated with 100μM of acyclovir for a period 2.5 hours before being infected

with virus. A set of three wells were not pre-incubated with acyclovir before infection. Each set was infected with 1 MOI of virus for one hour. Then each well was rinsed four times with serum free medium to remove any remaining input virus that had not been absorbed by the cells. Then α-MEM media that contained 5% CS and 100μM of acyclovir was added back to the wells that had been previously incubated with acyclovir. To the cells that had not been previously treated with acyclovir, only α-MEM with 5% CS was added. Each well was incubated with a total of 2 mls of media. The infected cells were observed every twelve hours. The supernatant fluids from each well were harvested at 36 and 72 hours post infection. After the supernatant fluids were harvested at 36 hours post infection, the same specific type of media was placed back into each of the wells. The experiment was terminated at 72 hours post infection.

Immunohistochemistry. The presence of neuronal cells was verified by the use of immunohistochemistry. Immunohistochemistry was carried out using Neurofilament 160 (Mice IgG) as the primary antibody. A Rabbit FAB:HRP antibody was used as the secondary antibody. The substrate used was black DAB (3,3'-diaminobenzidine).

Differentiated P19 cells were plated on glass slides for at least six days. The culture media was aspirated off the cells. The cells were rinsed with PBS for approximately five minutes. After the cells had been rinsed, they were fixed with fresh acetone for a period of 2-4 minutes. The slides were removed from the acetone and washed at least three times with PBS and .2% Tween. Unspecific binding on the tissue was prevented by adding rabbit serum for 40 minutes, followed by a treatment of blocking buffer (PBS with just .001 grams of BSA). The primary antibody (Chemicon Neurofilament 160) was added to the slides overnight. The slides were placed inside a humidity chamber inside a 4ºC refrigerator. After allowing the slides to incubate

overnight, the slides were rinsed at least three times with PBS/Tween. Next, the F(ab')2 rabbit anti mouse IgG: HRP secondary antibody (Serotec STAR13B) was added to the slides and left undisturbed for a period of 30 minutes in a humidity chamber. The slides were rinsed at least three times with PBS/Tween. The slides were then placed back in the humidity chamber, and DAB (Vector Labs) was added for fifteen minutes. After the DAB incubation period, the specimens were rinsed in tap water for five minutes. The slides were then counter-stained with Nuclear Red and then dehydrated. After counter-staining had occurred, the cells were mounted with permount. Some differentiated P19 cells on slides were prepared the same way as described above; however, they were not exposed to antibody and were counter-stained with eosin and hemotoxylin.

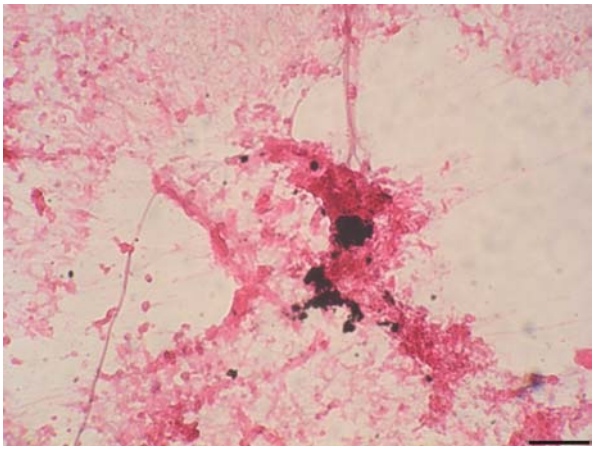

Figure 2.In this picture, the neurons are staining positive to Chemicon Neurofilament 160. The measurement bar represents 50μm.

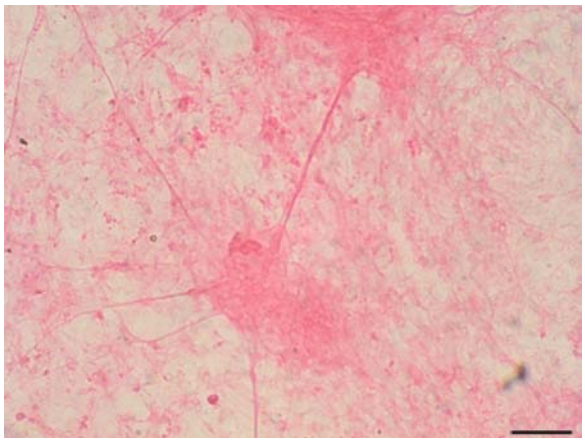

Figure 3.Control—Exposed to the same conditions as the stained cells above; however, primary antibody was not added.

Results & Discussion

The main purpose of these sets of experiments was to determine whether the four cell types tested were able to produce virus once infected with 1 MOI of HSV-1 virus. The amount of virus produced by each cell culture was determined by performing plaque assays on supernatant fluids taken from the infected cells. The hypothesis of this study is that CNS cell lines from the C3H mouse can be used as models to study HSV-1 viral production by different cell types, namely neurons, microglial cells, and a mixed cell population containing neurons, astrocyte-like cells, and fibroblast-like cells during a an active HSV-1 infection. P19 cells, the progenitor of the mixed brain cell culture and the isolated neuronal culture, was test as well. Only the mixed brain cell culture and the microglia cell culture supported the hypothesis.

P19 Cells

Plaque Assay Results

The highest number of viral plaques was detected at the 6 hour time point post infection. However, this was most likely residual virus left over from the input virus. After that specific time point, the viral titer declined. By the 48 hour time point, no virus was detected by the plaque assays.

These cells were not active producers of virus. The results were similar to the results obtained by John Bell et al. John Bell and colleagues infected P19 cells with the HSV-1 KOS strain. The P19 cells synthesized viral TK (an early beta gene product), gD, and viral DNA at levels similar to the levels seen in infected fibroblasts. However, very

few virion components were detected by electron microscopy. This suggests that HSV-1 replication is prevented at a late stage in the infectious cycle of the HSV-1 virus. In this study, the supernatant fluid samples were collected at the 24 hour time point after infection with HSV-1, strain 17+. By the 24 hour time point, the media in the cell culture contained no viable cells and a great deal of cellular debris was present. This is comparable to the observation by Bell et al. who noted complete CPE of P19 cells infected with the HSV-1 KOS strain at 20 hours post infection (Bell 1987). Many viruses that have enhancers that regulate gene expression have not been very successful in carrying out a productive infection in P19 cells. Therefore, viruses like HSV-1 that have enhancers may not be able to successfully replicate in embryonal carcinoma cells or other cells that cannot accommodate enhancers (Bell 1987).

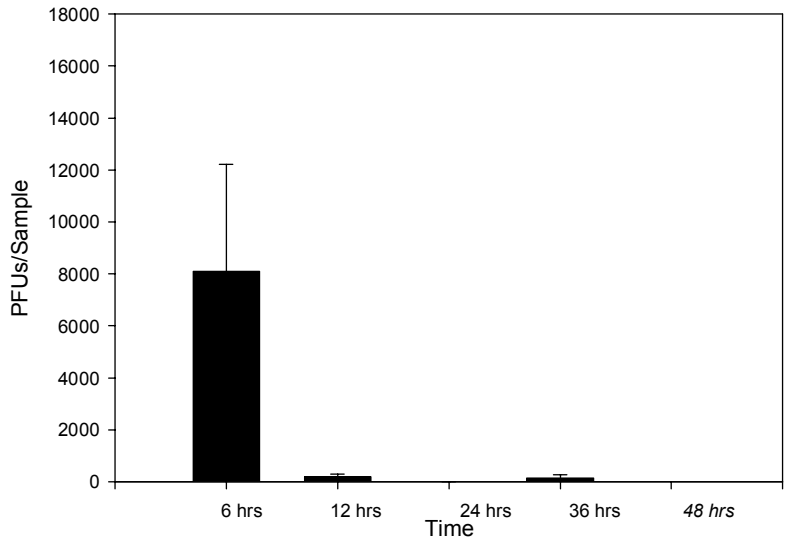

Figure 4.Illustrates the average viral titer present from infected P19 cell supernatant fluids at different periods post infection. The error bar is the standard error of the mean. The initial viral input was $2.5x10^5$ PFUs.

Isolated Neuronal Cells

Plaque Assay Results

Throughout this 48 hour assay, the viral titer declined. By the 48 hour time point, virus was not detected in the plaque assays. Due to the fact that the majority of the cells appeared to be dead at 12 hrs post infection and the viral titer declined, it is likely that these specific cells are not active producers of virus. These cells were very sensitive to the pathogenic effects of the virus. Because these cells were destroyed so quickly after infection, this particular cell culture would not serve as a good model to study latency or an active infection in neurons. The quick demise of these cells after infection could indicate that other central nervous system cell types such as astrocytes, fibroblast-like cells, or other support cells must interact with neurons to allow these neurons to survive infection and to experience latency. Because these neurons are derived from embryonal cell precursors and not more mature cells, these specific neurons may be ill equipped to survive an active infection. The artificial environment that these neuronal cells were placed in may also hinder their ability to survive.

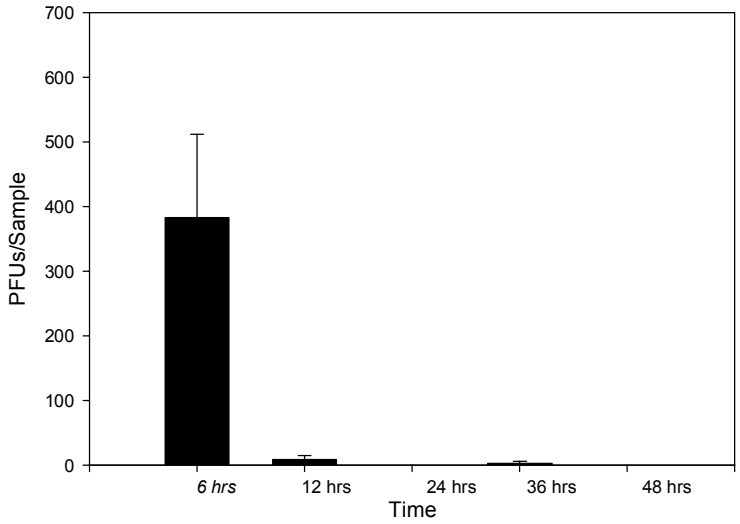

Figure 5. This chart illustrates the average viral titer from infected neuronal supernatant fluids at different times post infection. The sample bar is the Standard Error of the Mean. The viral input was 1.875×10^4 PFUs.

Mixed Brain Cell Types

Plaque Assay

Throughout the 6 and 12 hour time points post infection, the viral titer decreased. However starting at the 24 hour time point post infection, the viral titer began to increase. The increase in viral titer continued until the experiment was terminated at the 48 hour time point after infection. The virus that was present during the 6 and 12 hour time points was most likely residual virus that was left from the initial infection. However, an increase in viral titer after the first 24 hour time point was noticed. This increase was believed to be *de novo synthesis*.

After the virus attaches and penetrates the host cells, the eclipse period occurs. During the eclipse period, viral nucleic acids and viral proteins are being synthesized inside the cell. At this point, the virus is not released into the extracellular media. Once the virion particles have synthesized all of their needed components and are mature, then they are released to the outside where they can be harvested (Voyles 2002). Between 12 to 24 hours post infection, viral particles were released into the media outside of the infected cells. Therefore, it is likely that the eclipse period in these cells lasted until 12 to 24 hours post infection. Viral production continued to increase throughout the remainder of this experiment.

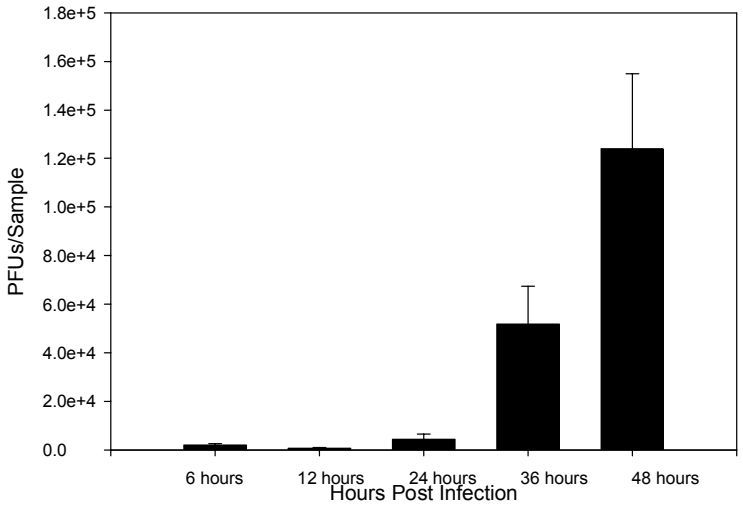

Figure 6.This bar graft represents the average HSV-1 titer for the supernatant fluids from the mixed brain cells taken at each time point. The error bar is the Standard Error of the Mean (SEM). The viral input was 6.425×10^5 PFUs. One way ANOVA was performed using Kruskal-Wallis One Way Analysis on Variance on Ranks $p < .050$.

The most likely cell types present in this mixed cell culture are neurons, fibroblast-like cells, and astrocytes. This experiment cannot determine the source of the virus production or if the cells present must interact with each other in order to produce virus. Future experiments could possibly be performed to determine if each specific cell type in the mixed culture produces virus independently.

A second experiment was implemented to confirm that *de novo synthesis* was indeed occurring in the mixed brain cell culture. The amount of virus produced by the mixed brain cell culture was lower than the amount of initial input virus. Therefore, another experiment was devised. This experiment was accomplished by studying two sets of wells. Three wells were only exposed to HSV-1; there was no substance added to impede *de novo synthesis*. Acyclovir was added to three other wells. Acyclovir is a potent anti-HSV-1 nucleoside analog that is incorporated into the growing strain of a HSV-1 virion. This incorporation prevents strands of HSV-1 DNA from becoming fully

and properly formed; therefore, *de novo* synthesis is greatly reduced. A total of six wells were tested. Wells A, B, and C were exposed to acyclovir 2.5 hours before infection and throughout the remainder of the experiment. Wells D, E, and F were not exposed to acyclovir. All wells were inoculated with 1 MOI of virus. The results are shown in the table below.

***Table 5. Experiment to Determine the Presence of *De Novo* Synthesis**

Well Type	36hrs	72 hrs
Well A	0.00E+00	0.00E+00
Well B	0.00E+00	0.00E+00
Well C	0.00E+00	0.00E+00
Well D	1.53E+04	1.13E+04
Well E	1.66E+04	8.67E+02
Well F	1.70E+04	9.22E+02

Legend
Wells A, B, and C were exposed to acyclovir and HSV-1.
Wells C, D, and E were exposed to only HSV-1.

*One way ANOVA was performed using Kruskal-Wallis One Way Analysis on Variance on Ranks p <.050.

No virus was detected with plaque assays during the 36 and 72 hour time points post infection in the wells that were exposed to acyclovir before and during the experiment. However, virus was detected in the supernatant fluids of the cells that were not exposed to acyclovir. The greatest amount of virus production was seen at the 36 hr time point. The amount of virus ($1.63x10^4$ PFUs) produced at 36 hrs in this experiment was slightly lower than the amount of virus ($5.9x10^4$ PFUs) produced by the previous experiment using mixed brain cells. However, the amounts produced in each experiment are within the same log. One of the supernatant fluids without acycovir had a much higher viral titer than the other two samples of supernatant fluids collected at 72 hours post infection. This may have been due to the fact that the mixed brain cell cultures in each of the three wells contained different cell types. In the event that one specific cell

produced the majority of virus and the components of one well had more of this specific cell type than the others did, the supernatant fluids collected from that well would most likely have a higher viral titer.

When the virus titers of the infected cells were compared with the virus titers from the cells exposed to acyclovir, it appears that acyclovir severely reduced the amount of *de novo synthesis* occurring in the infected cells. This last experiment proved two important things. First, it proved that *de novo synthesis* was occurring in the infected cells. If the mixed cell culture was not able to manufacture virions during the experiment, then the virion levels would be the same in both sets of wells. The last experiment also showed that this cell culture responded appropriately to acyclovir. In the experiment, acyclovir reduced viral titer. This is what occurs during an actual infection *in-vivo*. Other anti-HSV-1 drugs should be tested with this system to see if the model reacts to them the same way as what actually takes place *in-vivo*.

Microglia Cells

Plaque Assay

The microglial cell culture showed a decrease in viral titer during the 6 and 12 hour time points post infection. During this time it is likely that the only virus present was remaining input virus. Starting at the 24 hr time point, an increase in viral titer occurred. However, this increase was slight. The microglial cells were poor producers of virus. Microglial cells have been found to be poor producers of virus by other researchers.

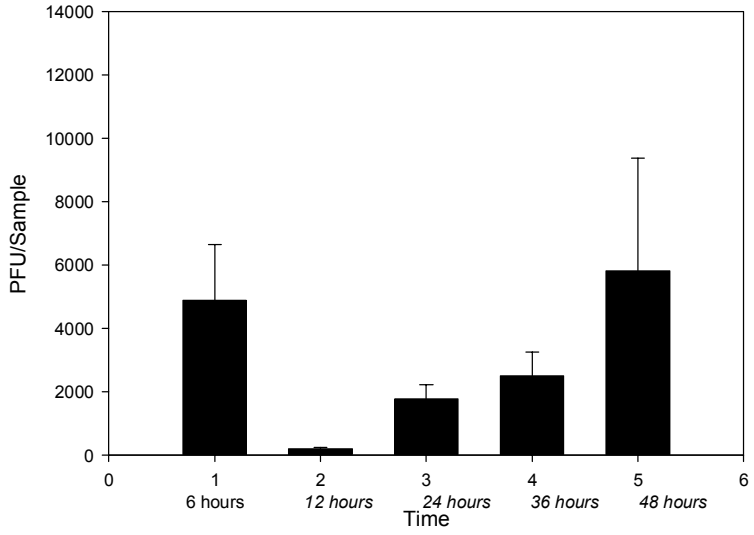

Figure 7.This bar graft represents the average HSV-1 titer for the supernatant fluids from infected migroglial cells taken at each time point. The error bar is the Standard Error of the Mean (SEM). The initial input was $2.5x10^5$ PFUs. One way ANOVA was performed using Kruskal-Wallis One Way Analysis on Variance on Ranks $p < .050$.

Assessment of Hypothesis and Future Research Suggestions

The only two models that support the hypothesis are the mixed brain cells and the microglial culture based on viral production. Microglia are present in humans of all age groups. However, the mixed culture is similar to only the human infantile stage of development or unmyelinated neurons in the adult brain. The neurons present in this cell culture are not myelinated nor do they have oligodendrocytes. The brain of an infant is mostly unmyelinated with only few oligodendrocytes. The mixed brain cells could possibly serve to model neural and neuralglial cells present in the brain of an infant (Arey 1974).

An adult brain has myelinated axons and oligodendrocytes present. This means that the mixed culture does not truly model the brain cells of an adult; unless a subpopulation of brain cells are studied. If a researcher wants to study adult brain cells

that are not myelinated and that are without oligodendrocytes, then this model may be appropriate (Arey 1974).

Previous *in-vitro* and *in-vivo* research has shown that neurons and astrocytes are producers of virus. The mixed brain cells produced virus during this experiment. However, additional tests are needed to determine which cell type/subgroups of cells are the true producers of the virus. This could be accomplished by viewing infected cells with electron microscopy at different time periods post infection. To date, the presence of neurons and astrocytes in the differentiated P19 culture has been verified with the use of immunohistochemistry and immunofluorescence (Jones-Villeneuve 1982). However, the cells that are similar to fibroblast-like cells need to be fully identified. Fibroblast cells are an integral part of the meninges which surrounds the brain. The role of fibroblast cells during an active infection in the CNS has yet to be fully explored. The exact nature of the fibroblast-like cells might also be determined by performing Real Time PCR on those cells and looking for mRNA that is produced only by fibroblast cells.

Lokensgard and colleagues showed that neurons and astrocytes were not the primary producers of cytokines during an actual infection. In order to determine if the neuronal cells and the astrocytes in the mixed culture produce cytokines, the remaining frozen supernatant fluids need to be analyzed for cytokine production. However, any cytokines produced by the fibroblast-like cells would also be detected.

Acyclovir has been shown to reduce virus load in animal experiments. Acyclovir reduced the viral titer when given to the mixed brain cells before and after infection. Therefore, the brain cells responded in a similar manner to brain cells *in-vivo*. However, it would also be beneficial to determine the effects of other antivirals on the mixed cell culture. If all of the additional experiments suggested here are performed and the results

mirror what actually occurs *in-vivo*, then the mixed brain cells may be able to serve as a model to study active infection in brain cells.

The microglial cells were also tested. They were not robust producers of virus. However, this mirrors what has been found in other research. Several experiments need to be performed in order to determine if the C3H microglial cell culture can be used as an effective model to study the effects of HSV-1 infection. It is crucial that the production of cytokines be examined in the supernatant fluids of infected cells. The response of these microglial cells to anti-virals should also be determined and compared to *in-vivo* models. Once this has been accomplished, one can better evaluate the use of this cell line to study HSV-1 infection.

Determining whether these cells are more effective models than *in-vivo* and primary cell *in-vitro* counterparts is also another important consideration when deciding if these cell cultures are to be effective models. Disadvantages exist in the use of these cell cultures to either test anti-virals or to study *in-vitro* co-cultures. There is no component in these models that mimics the blood brain barrier in the CNS. The blood brain barrier of the CNS is the main barrier the separates neural tissue from the blood. This barrier is composed of special endothelial cells that surround CNS capillaries and prevent the majority of molecules from passing into neural tissue from the blood. The astrocytes help to regulate the types and amounts of molecules that are allowed to pass into the neural tissue from the blood. For example, they regulate the concentration of K+ ions in the neural tissue. The blood brain barrier prevents many substances like antibiotics from entering the brain. Even if a drug or a molecule can affect the brain during *in-vitro* conditions, it may not actually affect the neurons and neuroglia in the CNS *in-vivo* because it cannot pass through the blood brain barrier (Kingsley 2000).

The absence of other organs and cell types that are in the body may cause results to be different when comparing data from *in-vitro* experiments with *in-vivo* experiments. In order to be effective, some drugs must be metabolized by certain organs before they can act on a target area of the body. It also follows that drugs that would normally be effective on an *in-vitro* system may not react the same way *in-vivo* when they are first modified by another organ. These continuous cell cultures do not show significant harm that drugs or therapies could cause to organs outside the central nervous system.

Lymphocytes and macrophages are not present in these cell cultures. Therefore, it would be impossible to determine what the full immune response would be during an active infection. This could possibly be corrected by isolating lymphocytes and macrophages and adding them to these cell lines.

Even though there are disadvantages associated with these continuous culture cell lines, there are many advantages that are not present in *in-vivo* models and *in-vitro* models comprising of primary cells. The absence of other cells such as the lymphocytes and macrophages allows for the study of the immune response between only a subset of cells. This cannot be accomplished with whole animal *in-vivo* models.

In-vivo models are very expensive because there must be funding available to house, feed, care, and medically treat animals used in research. The use of primary cells can accrue these types of costs to a lesser extent. Primary cells can only be cultured for a certain length of time before new cells have to be harvested from other animals. It also takes a great deal of time and equipment to extract the cells and prep them to be cultured. Continuous cells can initially be grown-up in media. They can be grown and frozen down indefinitely in a short period of time. The freezing down of stockpiles of continuous cells is much more cost effective than maintaining animals.

Both the microglial and the P19 cells (precursors of the mixed brain cells) can be easily purchased through American Type Culture Collection (ATCC). This allows researches to perform and collaborate on research using cells from the same source. Even if researches use the same strain of a specific animal when conducting *in-vivo* or *in-vitro* experiments, there may be some genetic variation present that could cause differences in data collected from different labs.

Unlike an actual *in-vivo* model, the process of infection can be continuously observed during an active infection using the C3H cell lines. The infection process of an *in-vivo* system is studied by sacrificing animals at various time points and observing regions of interest. In order to gain approval to use animals for *in-vivo* experiments or as the source of primary cells, researchers must first gain approval by using appropriate protocols and presenting compelling preliminary data. No such approval process is needed when using continuous cells from a reputable cell bank. In fact the results generated from experiments using these continuous cells could potentially be used as preliminary data that supports requests for experimentation using *in-vivo* animal models. Even though these two continuous cell lines have several limitations which would require testing to be performed using *in-vivo* models, these cell lines have characteristics analogous *in-vivo* CNS cells which could allow them to be used as models to study HSV-1 infection. However, before these cell lines can be used as models or in a co-culture system, the additional experiments mentioned above need to be completed.

Appendix A

Raw Data for the Infectivity Assays

P19 HSV-1 Viral Titer

Plaque Assays	Wells	PFUs/Well
6hrs		
PA-(6A/6B)	Well A	**6.13E+03**
PB-(6A/6B)	Well B	**2.19E+03**
PC-6B	Well C	**1.60E+04**
Average	Average	**8.11E+03**
12hrs		
PA-12B	Well A	**1.44E+02**
PB-12B	Well B	**4.00E+02**
PC-12B	Well C	**2.40E+01**
Average	Average	**1.89E+02**
24 hrs		
PA-24CF	Well A	**0.00E+00**
PB-24A	Well B	**1.60E+01**
PC-(24CF,24DF,24EF)	Well C	**0.00E+00**
Average	Average	**5.33E+00**
36 hrs		
PA-36DF	Well A	**0.00E+00**
PB-36A	Well B	**4.00E+02**
PC-36A	Well C	**3.20E+01**
Average	Well D	**1.44E+02**
48 hrs		
PA-48EF	Well A	**0.00E+00**
PB-48DF	Well B	**0.00E+00**
PC-48CF	Well C	**0.00E+00**
Average	Average	**0.00E+00**
Total Produced		**8.45E+03**

Neuronal HSV-1 Viral Titer

Plaque Assays	**Wells**	**Total PFUs**
6hrs		
NA-(6A/6B)	Well A	**5.76E+02**
NB-(6A/6B)	Well B	**1.40E+02**
NC-(6A/6B)	Well C	**4.32E+02**
Average		**3.83E+02**
12 hrs		
NA-(12A/12B)	Well A	**4.00E+00**
NB-12A	Well B	**0.00E+00**
NC-12A	Well C	**2.00E+01**
Average		**8.00E+00**
24 hrs		
NA-(24A/24B)	Well A	**0.00E+00**

NB-24A	Well B	**0.00E+00**
NC-24A	Well C	**0.00E+00**
Average		**0.00E+00**
36 hrs		
NA-36A	Well A	**0.00E+00**
NB-36A	Well B	**8.00E+00**
NC-36A	Well C	**0.00E+00**
Average		**2.67E+00**
48 hrs		
NA-48B	Well A	**0.00E+00**
NB-48B	Well B	**0.00E+00**
NC-48A	Well C	**0.00E+00**
Average		**0.00E+00**
Total Produced		**3.93E+02**

Mixed Cell Culture HSV-1 Viral Titer

Plaque Assay	Wells	Total PFUs
6 hrs		
MA-6B	Well A	**7.20E+02**
MB-6B	Well B	**3.36E+03**
MC-6B	Well C	**1.52E+03**
Average		**1.87E+03**
12 hrs		
MA-12A	Well A	**2.40E+02**
MB-12A	Well B	**9.60E+02**
MC-12A	Well C	**7.20E+02**
Average		**6.40E+02**
24 hrs		
MA-(24CF/24DF)	Well A	**4.20E+02**
MB-24CF	Well B	**7.28E+03**
MC-24BF		**5.60E+03**
Average		**4.43E+03**
36 hrs		
MA-36A		**2.08E+04**
MB-36A		**6.76E+04**
MC-36A		**6.72E+04**
Average		**5.19E+04**
48 hrs		
MA-(48A/48B)		**1.54E+05**
MB-(48A/48B)		**1.56E+05**
MC-48B		**6.24E+04**
Average		**1.24E+05**
Total Produced		**1.83E+05**

Microglia HSV-1 Viral Titer

Plaque Assay	Wells	Total PFUs

6hrs		
MiA-(6A/6B)	$8.35x10^3$	**8.35E+03**
MiB-6B	$2.48x10^3$	**2.48E+03**
MiC-6B	$3.84x10^3$	**3.84E+03**
Average		**4.89E+03**
12 hrs		
MiA-(12A/12B)	$2.82x10^2$	**2.82E+02**
MiB-(12A/12B)	$1.76x10^2$	**1.76E+02**
MiC-(12A/12B)	$1.8x10^2$	**1.80E+02**
Average		2.13E+02
24 hrs		
MiA-(24A/24B)	$1.33x10^3$	**1.33E+03**
MiB-(24A/24B)	$1.36x10^3$	**1.36E+03**
MiC-(24A/24B)	$2.66x10^3$	**2.66E+03**
Average		**1.78E+03**
36 hrs		
MiA-36B	$1.76x10^3$	**1.76E+03**
MiB-36B	$1.76x10^3$	**1.76E+03**
MiC-36A	$4x10^3$	**4.00E+03**
Average		**2.51E+03**
48 hrs		
MiA-(48A/48B)	$3.6x10^3$	**3.60E+03**
MiB	$1.04x10^3$	**1.04E+03**
MiC-48B	$1.28x10^4$	**1.28E+04**
Average		**5.81E+03**
Total Produced		**1.52E+04**

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