MECHANISMS RESPONSIBLE FOR THE DISPARATE PATHOGENICITY OF *HERPESVIRUS PAPIO* 2 SUBTYPES IN MICE

By

KRISTIN MICHELLE ROGERS

Bachelor of Science Microbiology
Oklahoma State University
Stillwater, Oklahoma
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Dissertation Approved:

Dr. R. Eberle

Dissertation Adviser

Dr. Ulrich Melcher

Dr. Jean d'Offay

Dr. Jeremiah Saliki

Dr. A. Gordon Emslie

Dean of the Graduate College

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NOMENCLATURE

BHV-1 and -5	bovine herpesvirus type-1 and -5
BSL	biosafety level
BV	monkey B virus (*Cercopithecine herpesvirus* 1)
CD_{50}	50% clinical disease dose
CNS	central nervous system
$CNSD_{50}$	50% central nervous system disease dose
EHV-1 and -4	equine herpesvirus type-1 and -4
e.s.	eye splash inoculation
H&E	haematoxylin and eosin
HSV-1 and -2	herpes simplex virus type-1 and -2
HVP2	*Herpesvirus papio* 2 (*Cercopithecine herpesvirus* 16)
HVP2ap	apathogenic subtype of HVP2 identified in mice
HVP2nv	neurovirulent subtype of HVP2 identified in mice
HVS-1	*Herpesvirus saimiri* 1
i.c.	intra-cerebral inoculation
ID_{50}	50% infectious dose
IFN-α and –β	interferon alpha and interferon beta
IHC	immunohistochemistry
i.m.	intra-muscular inoculation
Kbp	kilobase pairs
LD_{50}	50% lethal dose
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post-infection
PMDF	primary mouse dermal fibroblasts
PNS	peripheral nervous system
PRV	pseudorabies virus
RNA	ribonucleic acid
SA8	simian agent 8 (*Cercopithecine herpesvirus* 2)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.s	skin scarification inoculation
UL	unique long
US	unique short

FOREWARD

This dissertation includes three peer-reviewed scientific publications (Chapters IV, V, and VI) which appear with the journals' permission in the published journal format. As primary author, I performed the majority of experimental work as well as wrote and revised these manuscripts for publication.

1. Rogers, K. M., R.F. Wolf, G.L. White, and R. Eberle. 2005. Experimental Infection of Baboons (*Papio cynocephalus anubis*) with Apathogenic and Neurovirulent Subtypes of *Herpesvirus papio* 2. Comp Med 55:425-430.

2. Rogers, K. M., J.W. Ritchey, M. Payton, D.H. Black, and R. Eberle. 2006. Neuropathogenesis of *Herpesvirus papio* 2 in Mice Parallels *Cercopithecine herpesvirus* 1 (B Virus) Infections in Humans. J Gen Virol 87:267-276.

3. Rogers, K. M., D.H. Black, and R. Eberle. *In press*. Primary Mouse Dermal Fibroblast Cell Line as an *In Vitro* Model System for the Differential Pathogenicity of Cross-Species *Herpesvirus papio* 2 Infections. Arch Virol.

CHAPTER I

INTRODUCTION

With the realization that many new or newly recognized emerging infections of humans are zoonotic in origin, most now recognize the importance of basic research investigations into the mechanisms of cross-species or zoonotic viral transmission and pathogenesis. Although the zoonotic nature of human rabies infection was first described in the pre-Mosaic Eshmuna Code of Babylon written in the 23rd century BC, human rabies infections are still prevalent today (1). More recently, closely related lyssaviruses have resulted in human infection when humans are bitten or scratched while handling fruit-eating bats (2). Infections with Marburg virus and Ebola virus cause a fulminant hemorrhagic disease associated with high mortality in humans and other non-human primates (14). Within North America, hantavirus and West Nile virus are both responsible for human infections when transmitted from their natural hosts, small rodents and birds, respectively (16, 22). Finally, transmission of simian immunodeficiency virus into humans from chimpanzees (5) has been suggested to be the origin of the human immunodeficiency virus which has now reached pandemic status within the human population. Factors such as human population demographics, changes in lifestyles, advancing technology, and changing agricultural practices continue to provide novel opportunities for infectious agents to enter the human population from hosts previously thought to be outside of the range of "normal" contact.

Cercopithecine herpesvirus 1 (monkey B virus; BV) is an alpha-herpesvirus indigenous to macaques. In its natural host species, BV is biologically similar to other alpha-herpesviruses such as herpes simplex virus (HSV) types 1 and 2 in humans. Animals acquire BV either as an oral or genital infection of mucosal epithelial cells, and the virus establishes latency in sensory ganglia (7, 11, 12, 17). Similar to disseminated HSV infections in human neonates or the immune compromised (21), serious or fatal disease resulting from BV infection of the natural host most often occurs in either the young or immunocompromised adults (23). A quite different scenario arises when BV is transmitted to humans or other non-macaque primates: BV infection results in rapid invasion and destruction of the central nervous system (CNS) (6, 9, 11, 17, 20, 23). The necessity and demand for macaques in biomedical research has led to increased numbers of macaque colonies coincident with several human BV exposures and infections. Although human BV infections are sporadic, a fatality rate that exceeds 70% makes BV the major zoonotic concern for personnel who routinely handle macaques (11). Due to the extreme danger associated with BV in humans, research investigations into these cross-species infections are further complicated by the fact that BV is the only herpesvirus to be classified as a biological safety level 4 (BSL-4) pathogen. In addition, the U.S. government has designated BV as a 'select agent' due to concern regarding its use as a potential weapon of bioterrorism.

Infection of non-natural host species with other primate alpha-herpesviruses has resulted in various outcomes. The squirrel monkey alpha-herpesvirus (*Herpesvirus saimiri* 1; HVS-1) has produced severe and often fatal infections in both owl monkeys and marmosets (8, 10, 13, 15). Further, in BALB/c mice, experimental infection via

epidermal scarification with HVS-1 results in severe CNS infection (3, 4). In contrast to HVS-1, *Cercopithecine herpesvirus* 2 (simian agent 8; SA8) of vervet monkeys is apathogenic in mice (18).

Baboons are the natural host of the alpha-herpesvirus *Cercopithecine herpesvirus* 16 (*Herpesvirus papio* 2; HVP2). HVP2 is very closely related to both BV and SA8 at the biologic, genetic and antigenic levels (7). Although many people have been scratched and bitten by baboons, there is no evidence for human HVP2 infection. This lack of documented zoonotic potential coupled with the mild, self-limiting infections usually seen in the natural baboon host, has provided very little motivation for investigations into HVP2. However, two different strains of HVP2 were recently shown to rapidly invade the CNS of mice after intra-muscular inoculation to cause a fulminant, fatal, ascending encephalomyelitis equivalent to infections induced by the most virulent strains of BV infection in mice (18). Additional HVP2 strains were subsequently shown to produce neither clinical signs nor histopathological evidence of infection following intra-muscular inoculation of mice, similar to infection of mice with SA8. HVP2 was subsequently divided into two distinct subtypes based on their neuropathogenic phenotype in mice: HVP2nv (neurovirulent) and HVP2ap (apathogenic) (19). Phylogenetic analysis of a limited region of the HVP2 genome confirmed the existence of two distinct subtypes of the virus (19).

Observations on the pathogenicity of primate alpha-herpesviruses in non-natural host species leads to the question at the core of viral zoonoses: Why are some alpha-herpesviruses capable of producing extreme, often fatal CNS infections when they jump

species while other very closely related viruses either do not infect non-natural hosts or produce only very mild, clinically inapparent infections when they do? The disparate pathogenicity of HVP2 subtypes within a single genetically identical host species (ie. BALB/c mice) provides the opportunity to examine the viral mechanisms responsible for the extreme neurovirulence of alpha-herpesviruses following transmission to a non-natural host. The biological similarity and close genetic relationship of the two viruses (HVP2 and BV) substantiates the use of the BSL-2 agent HVP2 as a safe, effective system to model infections of the more hazardous BV. An added benefit of the HVP2 model is that the biology and pathogenesis of HVP2 can also be experimentally investigated in the natural baboon host. The focus of this dissertation is the elucidation of the genetic differences between HVP2nv and HVP2ap that underlie the dichotomous pathogenicity observed in BALB/c mice utilizing DNA sequencing/analysis and construction of intra-typic HVP2 recombinants.

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CHAPTER II

REVIEW OF LITERATURE

The Family *Herpesviridae*

Viruses in the family *Herpesviridae* are extremely prevalent in nature, with all vertebrate animal species and at least some invertebrates being host to at least one herpesvirus. Herpesviruses appear to be extremely ancient, possibly hundreds of millions of years old. It is thought that mammalian, avian, and reptilian herpesviruses all descended from a common ancestor (221, 225). Evidence suggests that these viruses have co-evolved with their natural host species to reach an exquisite level of symbiotic harmony (223, 226).

Inclusion in the *Herpesviridae* family is based on the architecture of the virion. Herpesvirus virions vary in size from 120 to nearly 300 nm and consist of four components: a core containing 120-160 kbp of linear double stranded DNA, an icosadeltahedral capsid, asymmetrically distributed globular material designated as the tegument, and a cell-derived lipid bilayer membrane envelope containing glycoprotein spikes (285). The base composition of the herpesvirus genome varies between species, ranging between 31-75% G+C, and these viruses have 70-200 genes.

While the *Herpesviridae* family includes viruses which exhibit wide variation in some viral properties, several key attributes unify members of the family: 1) the production of infectious herpesvirus progeny virus results in the irreversible destruction

of the infected cell; 2) viral DNA replication and virion assembly occur in the nucleus; and 3) herpesvirus genomes can exist in an episomal form within their natural host for the life of the host. Members of the *Herpesviridae* family are divided into sub-families designated alpha, beta, gamma, and delta based on biological properties such as host range, duration of reproductive cycle, cytopathology, and the neurotropism of latent infections (63, 284).

Alphaherpesvirinae

Members of the *Alphaherpesvirinae* subfamily are characterized by a highly variable host range, a short reproductive cycle, rapid spread in cell culture, a lytic infection, and establishment of a latent infection primarily in the neurons of sensory ganglia (284).

Humans are the natural host to three alpha-herpesviruses: herpes simplex virus type -1 (HSV-1) and -2 (HSV-2), and varicella zoster virus (VZV). HSV-1 is the prototypic member of the genus *Simplexvirus*. HSV-1 infects approximately 80% of the U.S. population and usually manifests as ulcerative orolabial lesions. HSV-2 is primarily associated with anogenital lesions and approximately 22% of the adult American population is seropositive for HSV-2 (362). HSV infections are responsible for a wide range of clinical manifestations in humans ranging from localized mucocutaneous infections to the more rare cases of uncontrolled, peripheral dissemination and fatal infections of the CNS. Neonates and immunocompromised individuals have a higher risk for developing the more serious complications.

VZV, a member of the genus *Varicellovirus,* is responsible for a wide range of acute and chronic disorders in the human population. The acute infection primarily manifests as chickenpox followed by a latent infection established in the cranial nerve and dorsal root ganglia. Frequently, latent VZV reactivates decades later to produce shingles, a zosteriform infection, and/or postherpetic neuralgia. In some cases, following reactivation the virus spreads to the CNS where it is capable of producing a wide range of clinical disorders including myelitis, meningitis, and encephalitis (122). Spread of VZV into the CNS is most common in immunocompromised patients.

Other alpha-herpesviruses such as porcine pseudorabies virus (PRV) and bovine herpesvirus (BHV) type 1, and to a lesser extent type 5, have also been well studied due to the economic impact these viruses have on the agricultural industry. Additional animal pathogens that impact agriculture include equine herpesvirus (EHV) -1 and -4, and avian herpesviruses such as Marek's disease virus and infectious laryngotracheitis virus, both of chickens.

PRV, a member of the genus *Varicellovirus*, is the causative agent of Aujeszky's disease in swine. While it has a very broad host range including birds and many non-primate mammals, the pig is the only host that can survive a productive infection and remain latently infected (232). Primary viral replication occurs in the nasal and oropharyngeal mucosa (183). Morbidity and mortality associated with PRV infection varies with age and health status of the host as well as viral strain and inoculum dose. Young swine are the most severely affected by PRV infection and typically exhibit

symptoms of CNS disease whereas PRV primarily manifests in older pigs as a respiratory disease (183).

While both BHV-1 and BHV-5 are neurotropic in cattle, BHV-5 is neurovirulent, causing fatal encephalitis in calves. BHV-1 is generally non-neurovirulent and is responsible for abortions, respiratory infections, and genital infections. Following primary infection of the eye and/or upper respiratory tract, BHV-1 is transported to, and establishes latency within, the trigeminal ganglionic neurons. As described for other viruses, stress can induce reactivation of the virus. BHV-1 can be transmitted to goats, both experimentally (305, 346) and in a natural setting (333). Although primary BHV-1 infection of goats is clinically inapparent, a latent infection capable of reactivation is established in these cross-species infections. In addition, red deer were susceptible to high dose inoculations of BHV-1 while reindeer were refractory to infection even at substantial viral doses (248). Finally, natural BHV-1 infections and experimental BHV-5 infections in sheep have also been reported (13, 302, 337).

The horse is the natural host of five known alpha-herpesviruses with EHV-1 and EHV-4 being the most clinically and economically relevant pathogens (60). Until 1981, EHV-1 and EHV-4 were considered to be two subtypes of the same virus (290, 293). While EHV-4 is primarily associated with respiratory disease, EHV-1 is more frequently associated with abortions, foal morbidity, and neurologic disease (258). Recently, the neurological sequelae resulting from EHV-1 infections have become more common and more severe. Recent outbreaks of EHV-1 induced CNS disease have resulted in considerable losses to the equine industry (42, 113, 321, 341). In addition to horses,

EHV-1 infections have been diagnosed in various equine species in captivity including Przewalski's horse, Burchell's zebra, Grevy's zebra, and Persian wild ass (236, 360). Restriction endonuclease profiles of EHV-1 isolates from captive zebras and onagers indicate that these strains are related to EHV-1 but differ slightly from EHV-1 isolates obtained from domestic horses (360).

Alphaherpesvirinae Biology

The genomes of viruses in the *Alphaherpesvirinae* subfamily are composed of two regions of unique sequence called the unique short (US) and unique long (UL) regions based on their length relative to one another (Figure 2.1). Genes in the unique regions exist in a single copy and are generally numbered according to their position from left to right in the prototype arrangement of the genome (UL 1-56 and US1-12). However, it is not uncommon for viral genes to be described by alternate names based upon their function. Both the UL and US regions are flanked by inverted repeat sequences which are either terminal (TRL or TRS) or internal (IRL or IRS) (Figure 2.1). These repeat sequences contain coding sequence for viral genes that exist as diploid genes as well as reiterated sequences. One set of reiterative sequences, designated the 'a' sequence, can exist internally in inverted form, repeated at one end, or repeated at both ends. This allows multiple isomeric forms of a genome to be packaged into virions (345).

Due to the large genome size, herpesviruses can encode numerous proteins which facilitate the infection process at multiple steps including viral entry and egress, gene expression, viral DNA replication, and virion assembly. As such, these genes are essential for infectivity and replication both *in vitro* and *in vivo*. Overall, essential genes

are defined by their necessity *in vitro* and code for structural proteins, proteins necessary for viral gene expression, or viral DNA replication/genome packaging. The remaining non-essential viral genes likely fill some specific niche in the course of an *in vivo* infection to enhance viral gene expression and/or overcome the host's response to the infection.

A defining characteristic of all alpha-herpesviruses is the coordinately regulated cascade of viral gene expression that occurs in temporal classes: immediate early (IE), early (E), and late (L) genes (152). Further, all viral genes are transcribed in the nucleus of infected cells utilizing the host RNA polymerase II (8, 58).

Expression of IE viral genes occurs in the absence of *de novo* viral protein synthesis via recruitment of cellular transcription factors to the promoters of viral IE genes. Gene products expressed with IE kinetics include regulatory proteins which are responsible for promoting E and L gene expression as well as manipulation of the host cell to create an environment suited for viral replication (164, 268, 349). In addition, certain IE gene products inhibit the expression of the IE genes to coordinate the switch from IE to E gene expression (101, 190).

Because herpesviruses often infect non-dividing cells, these viruses must encode enzymes involved in nucleic acid metabolism and DNA synthesis. Thus, the products of the E genes are primarily involved in DNA replication and must be expressed prior to the start of viral DNA replication. The promoter region for E viral genes may contain binding sites for cellular transcription factors and these DNA/protein interactions are activated by viral IE gene products (43, 308). As part

of the cascade, some E proteins are also required for the transcription of late genes to begin (116).

Viral genes expressed with late kinetics are transcribed only after viral DNA replication has occurred. Proteins in this class include structural proteins and several key proteins which are packaged in the tegument of newly formed virions for immediate release into newly infected cells.

Replication of alpha-herpesvirus DNA occurs in specialized compartments within the nucleus of infected cells (270). Circular viral DNA and the viral proteins necessary for DNA replication localize to cellular structures called nuclear domain 10 (ND10) sites (158). ND10 structures are subnuclear spherical structures which represent accumulations of multiple cellular proteins. Key proteins which comprise these ND10 structures include transcriptional activators, repressors of gene expression, and the promyelocytic leukemia (PML) protein (246).

Viral DNA replication initiates at an origin of replication (ori) located either in the short repeats (oriS) or the UL (oriL) component of the genome. Initially replication of the viral genome proceeds through a theta mechanism, but later changes to a sigma or rolling circle mechanism which produces head-to-tail concatemers of the viral genome (287, 300, 371).

The capsid proteins are synthesized in the cytoplasm, and transported to the nucleus for assembly either by using their own nuclear localization signal (NLS) or by binding to another protein containing an NLS (185, 281). Once capsids have

assembled, the HSV UL6 protein has been shown to assemble into ring-like structures *in vitro* that may form a portal for entry of viral DNA into the capsid (249). Viral DNA concatemers are cleaved at precise locations to release unit length genomes into the preformed capsids (288). Two herpesvirus-conserved *cis*-acting elements, *pac1* and *pac2*, are necessary for the combined process of cleavage and packaging (65-67, 137, 244). While the exact mechanism of *pac* –directed cleavage and packaging has yet to be determined, the current model suggests that *pac2* elements impart directionality to concatemer packaging by binding proteins that initiate insertion of concatemer ends into empty capsids (227).

Alpha-Herpesvirus Infection

Alpha-herpesviruses have two modes of infection in their natural host species: productive and latent. During productive infections, infectious progeny virions are produced and released at the expense of the infected host cell. Productive infections occur within epithelial cells at the initial site of inoculation during either the primary or recurrent disease stage. In contrast, latent infections result from persistence of the inactive viral genome within the dorsal root ganglia of sensory nerve endings that serve the initial epithelial site of infection (285, 286).

In a primary productive infection, incoming virus infects epithelial cells. Once in the epithelium, the virus replicates and releases progeny virions which infect and destroy neighboring cells. Viral gene expression during productive infection involves activation of the complete set of herpesviral genes in the aforementioned cascade of viral gene expression. During primary infection, virus enters sensory nerve endings at the

epithelial site of infection for transport via retrograde axonal flow to sensory ganglia in the peripheral nervous system (PNS) (286, 289).

Due to the lytic nature of the viral infection, cells at the site of entry as well as some sensory neurons are destroyed by the productive infection. However, in some sensory ganglia the virus establishes a latent infection that can persist in a nonproductive state for the rest of the life of the host. From time to time the virus can be reactivated by such factors as stress, UV light, or fever. Reactivated virus travels via sensory nerves back to the original epithelial site of infection. Viral replication in the epithelial tissue provides for the release of infectious virus with or without clinically apparent recurrent lesions at the original epithelial site of infection.

Garcia-Blanco et al. defined latency as " ... the reversible, non-productive infection of a cell by a replication-competent virus" (118). This definition clearly differentiates latent infections from persistent infections in which the virus maintains a productive infection by constant, low-level, viral replication. The fact that the virus only enters latency in neurons suggests that the virus-cell interactions within neurons are very different from those that occur in other cell types. One hypothesis is that HSV establishes latency in neurons in which IE gene expression does not reach a certain threshold level required for the continuation of viral replication (112). Another hypothesis is that the synthesis of viral DNA is a key regulatory event that determines if the virus will initiate a lytic vs. a latent infection within infected neurons (250).

During viral latency, the viral genome remains in the neuronal nucleus in a circular, extra-chromosomal state and lytic gene expression is repressed (229, 282). The prevailing hypothesis over the past few decades has been that only the region of the viral genome coding for the latency-associated transcripts (LATs) is transcribed. These LATs are not polyadenylated and accumulate primarily in the nuclei of latently infected PNS cells. The full-length 8.3 kb primary LAT mRNA accumulates to low levels in latently infected neurons. From this primary LAT, 2.0 kb and 1.5 kb transcripts are produced. These smaller transcripts are more abundant and much more stable than the primary LAT mRNA (188, 316). Although there is neither a protein product nor an exact function associated with the LATs in the natural host, other activities for the LAT transcripts have been suggested. Viruses that do not produce LATs show increased IE gene expression, suggesting that the LATs may inhibit or limit lytic viral gene expression in favor of the establishment of a latent infection (117). It has also been suggested that LATs may act via an antisense mechanism because a portion of the 8.3 kb LAT overlaps with the coding sequence for two IE viral genes (46). An alternate view is that the LATs protect neurons from apoptosis during latent infection (4, 266). While mutants which do not produce LATs are able to establish and maintain latency, often these mutants are defective for reactivation (22, 336).

Recently, a new theory of viral gene expression during latency has emerged: low levels of certain viral proteins are produced in latently infected neurons and recognized by $CD8^+$ T cells which serve to control viral reactivation. One study demonstrated localization of $CD8^+$ T cells to neuronal cell bodies in the trigeminal

ganglia of humans with a history of HSV-1 reactivation (327). A second study demonstrated that mouse $CD8^+$ T cells present in trigeminal ganglia cell cultures recognized a viral glycoprotein (gB). Following recognition of gB, a response was initiated to preclude expression of a second glycoprotein (gH) which is expressed after gB in lytic infections (178).

Alpha-Herpesvirus Pathogenesis

A defining characteristic of herpesviruses is their ubiquitous nature. These viruses are very common, found in all population groups, and have a worldwide distribution (64). As a result of the symbiotic host-pathogen relationship formed through extensive co-evolution of herpesviruses with their natural host species, serious infection in the natural host is rare. Primary infection with most alpha-herpesviruses generally occurs in a subclinical to mild manner, usually in early infancy or adolescence with subsequent latent infections which persist for the life of the host.

Infections of the Natural Host

While the clinical signs, disease processes, and severity of infection associated with alpha-herpesvirus infections in their natural host species vary, in general the clinicopathogenesis of the oral, genital, ocular, disseminated, and encephalitic forms of infection are quite invariant. In fact, much of what has been learned about herpesvirus infections has come from investigations into natural herpesvirus infections of species other than humans.

Herpes simplex viruses are separated into two distinct biotypes: HSV-1 and HSV-2 (297). Although HSV-1 is most commonly associated with oral infections and HSV-2 is considered primarily genital in nature, both types can cause infections at both sites. The route of transmission, rather than tropism of the virus for a particular type of tissue will generally determine the nature of the infection: HSV-1 is spread most often by close, non-sexual contact while HSV-2 is transmitted sexually.

In primary HSV infection, shallow ulcers may form in the infected epithelial layer as a result of the lytic replication of the virus, but both HSV-1 and HSV-2 primary infections may be asymptomatic. As the host innate immune system responds, ulcers may be accompanied by a mild fever and lymphadenopathy (73). Oral HSV infections will establish latency in the trigeminal ganglia while genital latent infections are established in the sacral ganglia. Reactivation of these infections will cause the release of infectious virus from the original epithelial site of infection either with or without recurrent lesions. In addition to the oral or genital manifestations, HSV infections can occur at several other locations within the human host.

Herpes simplex keratitis (HSK) results from HSV-1 infection of the eye. Although primary infection is most often mild, severe corneal damage results from reactivation of latent virus and leads to corneal scarring, thinning, and neovascularisation (176). Herpetic whitlow is an infection of the hand involving one or more fingers. HSV-1 is the cause of approximately 60% of cases of herpetic whitlow, while HSV-2 is the etiological agent in the remaining 40% (123). As in other mucocutaneous herpetic infections, herpetic whitlow is initiated by viral inoculation of the host through exposure

to infected body fluids via a break in the skin, most commonly a torn cuticle. Herpes gladiatorum refers to a skin infection that occurs in adolescent wrestlers. It usually occurs on the head, most commonly the jaw area, and is easily spread during tight wrestling holds. Most commonly, herpes gladitorum infections are associated with HSV-1 (157).

Much less is known about the molecular biology of HSV infections in the CNS. Two important criteria allow for viral pathogenicity within the CNS: the ability of the virus to enter the CNS (neuroinvasiveness) and its ability to replicate within tissues of the CNS (neurovirulence). In experimental animal models, the neuroinvasiveness of a particular strain of HSV-1 correlates with the ability of the virus to initiate zosteriform disease (126). Zosteriform disease is the result of secondary epithelial lesions at a site distinct from the initial site of inoculation but within the same dermatome. Lesions result from viral replication within neurons of the PNS followed by anterograde axonal transport of the virus back to the epithelium (304). Clinical syndromes associated with HSV CNS infection in humans generally fall into three categories: disseminated neonatal infection, encephalitis, or meningitis.

Neonatal HSV infection is primarily caused by HSV-2 transmitted from mother to infant either *in utero* or intrapartum via infected genital secretions (57). There are three syndromes associated with neonatal HSV disease: skin, eye and mouth (SEM) infection, CNS infection, and disseminated infection. SEM is rarely fatal but can lead to neurological impairment including quadriplegia and blindness (110). Untreated neonatal CNS infections have a mortality rate of 50% with a bleak long-term prognosis for survivors. Disseminated infection leads to encephalitis in about 60-75% of cases if not

treated immediately. The mortality rate of these infections exceeds 80% and nearly all survivors are severely neurologically impaired (354).

HSV encephalitis, most often the result of HSV-1 infection, is the most common cause of sporadic fatal encephalitis in non-infants with an incidence of 1 case per every 250,000-500,000 persons each year in the U.S. (127). HSV meningitis is a self-limiting illness commonly caused primarily by HSV-2 infection that occurs more frequently in young adult women than in young adult men with a ratio of 6:1 (275).

Alpha-herpesvirus infections in other species occur in a manner similar to HSV infection in humans. PRV is acquired through direct contact with the saliva or nasal discharge of infected swine. Clinical signs of infection in adult pigs are generally limited to fever and respiratory disease. Sows that become infected in gestation may reabsorb their fetuses, abort, or give birth to weak or defective piglets (231, 232). Infection of young piglets often results in severe CNS disease and sudden death.

BHV-1 is the etiological agent in numerous syndromes of cattle: infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious balanoposthitis, conjunctivitis, enteritis, encephalitis, and mastitis (317). The course of disease is determined by both the route of entry (respiratory vs. genital) and the age of the infected animal.

BHV-5 of cattle is primarily acquired as an upper respiratory infection in young calves, presenting as a mild rhinitis and conjunctivitis. Some animals are able to clear the virus and recover while others proceed to neurological disease and death (87, 265). In

addition, BHV-5 can cause abortion in pregnant females (87). Again, the age and immune status of the infected host are critical factors that determine the outcome of infection with BHV-5.

EHV-1 of horses is is acquired as a respiratory infection such that initial viral replication occurs in epithelial cells of the upper respiratory tract and local lymph nodes (259). Acute respiratory disease due to EHV-1 is characterised by fever, anorexia, nasal discharge, and ocular discharge. This initial infection may be followed by viremia and transport of the virus to endothelial cells in blood vessels of the CNS producing vasculitis and thrombosis which progresses to ischaemia and haemorrhage (259, 267, 315). This is in contrast to other alpha-herpesviruses which directly infect neurons and glial cells in the CNS. As such, the clinical signs of EHV-1 CNS infection due to EHV-1 include ataxia and paraplegia and differ from the characteristic signs of encephalitis observed with most other alpha-herpesvirus infections of the CNS (24).

Alpha-Herpesvirus Infections in a Non-Natural Host

When herpesviruses infect a susceptible non-natural host, very severe and often fatal infections of the CNS can occur. There are several key points in the infection process at which cross-species infections differ from infections of the natural host. The first difference is likely to occur at the initial site of infection. The virus must be able to replicate sufficiently at the site of inoculation for entry into the sensory nerve endings. Using a mouse model, Yamada et al. demonstrated a direct positive correlation between efficient replication at the site of inoculation and neurovirulence leading to the development of CNS disease (363). In contrast, a second set of

experiments showed that intra-nasal inoculation of HSV in mice produced an asymptomatic local infection which "primed" the innate immune system within the CNS so that the brain was protected from subsequent intra-cranial inoculation challenge with the same viral strain (7).

A second but correlated difference occurs in the early, rapid host innate immune response to the viral infection at the site of inoculation. Due to the high degree of co-evolution that exists between the natural host species and the virus, the natural host is well-equipped to respond rapidly and effectively limit viral replication. In contrast, a non-natural host species may not be equipped to handle the invading virus as well as the natural host. This could lead to increased replication at the site of infection which would amplify the quantity of virus that enters the sensory nerve endings and neuronal cell bodies of the PNS.

The increased input of virions into the PNS may help set the stage for the subsequent differences observed between infections of the natural vs. non-natural host. In the natural host, several days of viral replication within the PNS are followed by the cessation of virus production and establishment of a latent infection. When non-natural hosts are infected, viral replication in the PNS does not cease and a latent infection is not established. The continuation of viral replication coupled with the inflammatory nature of the immune response invariably leads to cellular death and tissue destruction. It may be that this destruction plays a role in allowing viral entry into the CNS, however, the exact mechanism of CNS invasion by alpha-herpesviruses remains unknown. In addition, the mechanisms of CNS invasion and virulence are

dependent on the host species and the particular alpha-herpesvirus. Therefore, while the mechanisms which contribute to neurovirulence may differ from virus to virus and host to host; examples which demonstrate the severity of cross-species alpha-herpesvirus CNS infections are numerous.

Severe cases of CNS infection have been reported in many non-human primates after natural HSV infection. Localized, benign HSV infections have been reported in chimpanzees, whereas systemic infections with fatal outcome have been described in gorillas, white-handed gibbons, white-faced saki monkeys, owl monkeys, and common marmosets (140, 153, 168, 217, 219, 228, 272, 298, 309). While severe gingivostomatitis and meningoencephalitis are the most prominent symptoms, disseminated infections involving the digestive tract, lungs, liver, and adrenal glands have also been reported (228, 298).

In addition to infections in its natural swine host, PRV can infect cattle, sheep, dogs, cats, goats, chickens, raccoons, opossums, skunks, rodents, rabbits, guinea pigs, and, rarely, horses (109, 132, 179, 220, 262, 358). Infection of wild carnivores, such as bears and wild felines, has been linked to consumption of raw PRV-infected meat (39, 125, 367). In addition, experimental studies have shown that both rhesus macaques and marmosets are susceptible to PRV infection (89, 183). Infection of these non-natural hosts with PRV is uniformly lethal. With the exception of rare cases of pruritis, PRV is generally considered non-infectious to humans (260).

Innate Immunological Response to Herpes Infection

The host innate immune response to viral infection is a crucial factor in determining the pathogenicity of a virus within the context of an individual host. Mediators of innate immunity are charged with limiting both viral replication and spread at the initial site of infection. The large number of mechanisms that herpesviruses have evolved to counteract host innate immune responses substantiates the importance of these early interactions to the outcome of viral infection and corroborates the hypothesis of host-virus co-evolution. When these viruses jump species, differences in the constituents and nature of the innate immune response in the non-natural host species, relative to the natural host, may dramatically affect the host-pathogen relationship and result in a very different clinical outcome.

Mediators of innate immunity include monocytes/macrophages, dendritic cells, natural killer (NK) cells, and soluble factors such as complement, cytokines, and chemokines. In the very early stages of an acute herpesvirus infection, the type I interferons (IFNs) IFN-α and IFN-β are critical first responders to a viral infection (196, 213, 238, 240). It has been demonstrated that primary HSV infection in mice can be controlled by IFN-α/β alone without need of B, T or NK cells (344).

Two distinct type I IFN induction pathways have been characterized and studied in detail. The classical model is utilized by most cells of the body including fibroblasts, hepatocytes, and conventional dendritic cells (136). Within cells infected with HSV-1, double stranded RNA (dsRNA) intermediates are produced as a result of viral transcription (162, 187). Sensor molecules within the infected cell recognize the dsRNA

and respond by activating cellular transcription factors including interferon regulatory factor (IRF) 3, nuclear factor kappa B (NF-κB), and the AP-1 heterodimer formed by c-jun and c-fos. The cooperative action of these factors results in the homodimerization of phosphorylated IRF3. These homodimers then enter into the nucleus where they recruit the coactivators p300 and CREB-binding protein to initiate expression of IFN-β within infected cells (147, 323). The binding of soluble IFN-β to the IFN-α/β receptor on neighboring cells initiates a signal transduction cascade via phosphorylation events in the JAK-STAT pathway (62, 128). An immediate consequence of this signal transduction cascade is the expression of IRF7 (called the master regulator of IFN-α/β expression) within uninfected bystander cells. Together, IRF3 and IRF7 amplify the expression of IFN-β as well as induce expression of IFN-α in what is known as the amplification phase of the IFN response. This establishes an anti-viral state in cells at the site of infection so that infected cells are recognized and destroyed while uninfected cells become primed to resist viral infection.

In contrast to the classical pathway of IFN induction, a second pathway initiates type I IFN production in infected plasmacytoid dendritic cells (pDCs) found in lymphoid tissues and peripheral blood. Because pDCs constitutively express high levels of IRF7 they are often referred to as natural IFN producing cells. pDCs express primarily IFN-α in response to viral infection via toll-like receptors (TLRs) expressed in endosomes (16). HSV has been shown to trigger an innate immune response through interaction of the virion/virion components with TLR2 (191) and TLR9 (209). In the case of the HSV-2/TLR9 interaction, the ligand for the TLR appears to be double stranded CpG-rich DNA which is common in the genomes of most alpha-herpesviruses (159). Again, the result of

this pathway is induction of a protective anti-viral state within uninfected cells at the site of infection.

The mechanism for inducing the anti-viral state involves binding of type I IFN to IFN-stimulated response elements (ISRE) within the promoter region of the IFN-stimulated genes (ISGs). These ISGs produce mediator proteins which function at both the intra- and intercellular level to limit early viral replication and spread. To date, three IFN-induced antiviral pathways have been well characterized: protein kinase R (PKR) (356), the 2-5 oligoadenylate synthetase-RNaseL system (303), and the Mx proteins (135). Of these, PKR appears to be the major player in the cellular battle against herpesvirus infections. The antiviral effect of PKR stems from its ability to phosphorylate eukaryotic initiation factor (eIF) -2α, an elongation factor, and effectively shut-down host cell protein synthesis.

In addition to establishing an anti-viral state at the initial site of infection, type I IFNs are responsible for many downstream facets of the innate response to viral infection including inhibition of viral replication; activation of NK cells, macrophages, and lymphocytes; and increasing the expression of MHC molecules (145). The evolution of numerous herpesvirus genes that play a role in evading or counteracting the type I IFN response reflects the importance of these cytokines in the host innate immune response to these viruses (201). In a display of exquisitely fine-tuned host-virus co-adaptation, HSV-1 activates the host IFN pathway in the absence of *de novo* protein synthesis (240, 251) and then suppresses the IFN response after viral genes are expressed (240).

Due to the potent and often cytotoxic effects mediated by IFN, the induction and expression of these systems are tightly regulated by the host. One way in which herpesviruses counteract the detrimental effects of IFN is by manipulating cellular proteins responsible for regulating immune responses. The suppressors of cytokine signaling (SOCS) molecules constitute a negative feedback system of the JAK/STAT pathway (115). Specifically, SOCS1 and SOCS3 have been reported to inhibit the antiviral activity of type I and II IFN (6, 313). HSV-1 induces SOCS3 expression 1-2 hours post infection to suppress IFN production and signaling in response to the viral infection (364). This suppression of IFN via SOCS3 has been shown to be required for efficient replication and lytic infection by HSV-1 in numerous human cell lines (365). Recognizing that IFN is a powerful enemy, these viruses have evolved mechanisms to interfere not only with the antiviral effects of IFN but also its production in infected cells.

NK cells and natural killer T (NKT) cells along with interleukin-15 (IL-15) are a second set of host innate defenses that plays an important role in the immediate, non-specific control of viral infections. NK cells are large, granular, non-T cell lymphocytes while NKT cells are T cells that express the α/β T-cell receptor in addition to some of the cell-surface molecules found on NK cells (21, 210). Both NK and NKT cells are cytotoxic and capable of killing virus infected cells without prior sensitization (353). NK cells also release IFN-γ, a cytokine of the adaptive immune system that is critical for the activation of antigen-presenting cells (19, 20).

IL-15 is an IL-2 like cytokine produced by virus infected cells or monocytes/macrophages, and these two cytokines both bind the IL-2/IL-15β receptor

(11). IL-15 is responsible for the development, maturation, and activation of both NK and NKT cells (44, 130, 348). Enhanced NK cell activity as a result of IL-15 induction has been shown to be protective against HSV-1 *in vitro* and HSV-2 *in vivo* (3, 133, 338). Furthermore, IL-2/IL-15β receptor knockout mice are more susceptible to HSV infection (338). Thus, NK cells and IL-15 appear to be important elements of the innate host response to herpesvirus infections.

Virus infection of a cell immediately elicits the release of a variety of other pro-inflammatory cytokines by the infected cell as well as neighboring uninfected cells. In addition to IFN-α/β and IL-15, other important cytokines include IL-6, IL-8, TNF-α, IL-12, MIP-1α, MIP-2, and MCP-1 (238). Many of these pro-inflammatory cytokines act as chemotactic factors to recruit neutrophils and cells of the macrophage lineage to the site of infection. While neutrophils attack infected cells and release additional cytokines, macrophages process viral antigen and migrate to draining lymph nodes to initiate the adaptive immune response to the virus. In addition, release of pro-inflammatory cytokines by local infected and uninfected cells as well as infiltrating neutrophils eventually attracts T-cells to the site of infection.

The CNS has historically been considered an immunologically privileged site due to the existence of the blood-brain barrier (245). Further, resident cells of the CNS express only very low levels of critical effectors of the immune response including major histocompatability complex (MHC) molecules, adhesion molecules, and costimulatory molecules (301). However, recent evidence supports the CNS as an immunocompetent site in which sentinel activated T cells enter from the blood (142) and resident CNS cells

can be stimulated to participate in a strong immunological response (301). Astrocytes both produce and respond to a number of cytokines and chemokines: IFN-α/β, TNFα, IL-1,4,10, and 12 and MCP-1 (14). Microglia are the resident macrophage of the CNS and exhibit a number of functions common to other macrophages including cytokine/chemokine production, antigen presentation and phagocytosis (189). Recently, it was shown that human neurons express TLR-3 and can mount an immune response reacting to HSV-1 infection *in vitro* (269). In addition, mounting evidence suggests that $CD8^+$ T cells produce IFN-γ in response to viral proteins produced by latently infected neurons to prevent the reactivation of latent herpesvirus infections (177). Thus, rather than providing a safe haven for unchecked viral replication, the host CNS represents yet another battlefield for the virus and host to confront one another in the ongoing struggle for survival.

Virulence Genes in Alpha-Herpesviruses

HSV-1 and -2, PRV, and BHV-1 have all been the focus of vaccine design studies, and in the case of HSV and PRV, studies that use the virus as a vector to shuttle therapeutic agents directly to the CNS. The ability to alter these viruses so that they lose their neurovirulent phenotype while retaining neuroinvasiveness is vital to these studies. To this end, much work has focused on herpesvirus genes which are not essential for viral replication *in vitro*, but rather are responsible for amplifying viral neuroinvasiveness and neurovirulence *in vivo*.

(a) RL1 – ICP34.5

One of the best studied HSV virulence determinants is the diploid RL1 gene that codes for infected cell protein 34.5 (ICP34.5). To date, an RL1 open reading frame (ORF) has been identified only in HSV-1 (48), HSV-2 (224), and macropodid herpesvirus 1 (MaHV-1) of marsupials (131). However, the MaHV-1 ICP34.5 was determined to be homologous to the HSV ICP34.5 based on a sequence of 59 amino acids that shared 68% similarity with the carboxy terminus of the HSV-1 protein (131). Further, no ICP34.5 protein has ever been identified or characterized from MaHV-1.

Interestingly, the HSV-1 ICP34.5 C-terminal domain shows 83% identity with the corresponding domain of the cellular growth arrest and DNA damage protein (GADD) 34 which is expressed in non-myeloid CNS and PNS tissues (222). The GADD proteins are induced by DNA damage and stress *in vivo*. The function of these proteins is to suppress cellular growth during DNA repair to preclude stress responses that may result in apoptosis. The current hypothesis is that HSV "borrowed" this coding region to allow the virus to block a cellular response to infection and preclude total protein synthesis shutdown and apoptosis (138). This hypothesis may account for the absence of a readily identifiable ICP34.5 homologue in the non-human alpha-herpesviruses (184, 264, 339).

The HSV-1 ICP34.5 is composed of three distinct portions: an N-terminal 155 amino acids; a bridge unit with a strain-dependent number of proline-alanine-threonine (PAT) repeats; and 65 C-terminal amino acids (26). ICP34.5 is expressed as an early protein that precludes the total shut-off of protein synthesis by the host cell in response to herpes infection. ICP34.5 redirects the double-stranded RNA-dependent protein kinase

PKR so that the α-subunit of eIF-2 remains dephosphorylated, and protein synthesis continues (139). The requirement for ICP34.5 has been shown to be dependent on the cell type, cell cycle stage, and differentiation state of the infected cell (34).

In Vero and SK-N-SH cell lines, stark differences in ICP34.5 cellular distribution and neuroinvasiveness have been correlated to the number of PAT repeats in the bridge region (214). In general, the authors demonstrated that HSV-1 strains such as LP5 (PAT repeats = 22) targeted ICP34.5 almost exclusively to the cytoplasm and had a virulent phenotype in a mouse model, while strains such as KOS321 (PAT repeats = 3) were targeted to the nucleus and completely attenuated in mice. In strains with an intermediate number of PAT repeats such as KOS79 (PAT repeats = 11), ICP34.5 was found in both the cytoplasm and the nucleus. The same study showed that ICP34.5 binds its ligand, protein phosphotase I, and either concentrates or delivers it to specific sites, either nuclear or cytoplasmic, depending on the number of PAT repeats.

HSV RL1 deletion mutants can grow to very high titers in some cell lines and yet be avirulent after intracerebral inoculation into mice, suggesting that the lack of virulence comes from an inability of the virus to multiply in the CNS. To this end, 4-5 week old mice were inoculated intracerebrally with an HSV-1 RL1 deletion mutant (215). The mutant virus was able to express viral proteins, cause tissue damage at the site of infection, and infect neurons, astrocytes, oligodendrocytes and ependymal cells. They concluded that the virulence phenotype was due to a reduced ability of the virus to replicate rather than discrimination between cell types in the CNS. Another study showed that HSV-1 RL1 deletion mutants failed to prevent the total shut-off of protein synthesis,

thereby rendering the host cell non-viable for viral replication, and severely decreasing the viral yield (49). These mutants were completely avirulent in experimental murine and guinea pig models (355). More specifically, a mutant designed with deletion of a 63-residue section from the C-terminus of RL1 failed to preclude the shut-off of protein synthesis (47). In another study, HSV-1 mutants lacking both copies of the RL1 gene showed a 4-fold less replication in Vero cell culture and a 100,000-fold reduction in neurovirulence after intracerebral inoculation into mice (49). The ability to replicate in some cell lines and yet be avirulent after intracerebral inoculation into mice is also seen in HSV-1 mutants with small in-frame deletions across the RL1 gene which suggests that the RL1virulence factor requires a largely intact gene (289). The RL1 gene is absent in HSV-1 strain 17, which is apathogenic in mice, but present in HSV-1 strain F, which is neurovirulent in mice (291).

RL2 – ICP0

The RL2 gene codes for the multifunctional protein ICP0 in HSV and homologous proteins in other alpha-herpesviruses. In general, ICP0 is considered a promiscuous transactivator in that it activates all classes of viral genes as well as selected host cell genes in the absence of a single *cis*-acting element (94). Investigations have revealed a wide-ranging list of functions attributed to the ICP0 protein within infected cells. Extensive and sequential post-translational modifications may account for the varied functions performed by ICP0 at different times and/or locations within infected cells (2).

RL2 is a diploid gene comprised of three exons, making it one of the few spliced mRNAs in the alpha-herpesviruses. It consists of numerous functional domains including

a nuclear localization signal, a high-affinity self-interacting domain, and a RING finger zinc-binding motif (54, 91, 94, 97). In addition to these functional domains within the protein, unspliced mRNA from HSV-1 ICP0 intron number two has been identified as a repressor of transcriptional activation by ICP0, possibly by titrating a cellular factor away from ICP0 (93, 314, 350).

One objective of ICP0 is to regulate the steady-state levels of both viral and cellular proteins (25, 342). Further, evidence supports the role of ICP0 as a ubiquitin ligase responsible for degrading cellular proteins through an association with 26S proteasomes in infected cells (98, 203, 206). As a consequence of its ubiquitin ligase activity, ICP0 promotes the disaggregation of ND10 structures within infected cells (218). As both formation of ND10 structures and accumulation of ND10 components including PML are regulated by IFN (277), degradation of ND10/PML by ICP0 diminishes the ability of IFN to interfere with viral replication very early after infection. In addition, ICP0 can prevent the arrest of host cell protein synthesis later in infection by halting cleavage of rRNA induced by ISGs within infected cells (312). Finally, ICP0 blocks IFN production by inhibiting IRF3 and IRF7-mediated activation of ISGs in infected cells (201). The fact that HSV ICP0 null mutants are extremely sensitive to type I IFN *in vitro* supports the importance of this viral pathway of immune evasion (239).

While ICP0 is not required for viral replication, *in vitro* infections at a low MOI with HSV ICP0 null mutants results in viral titers reduced some 10-100 fold (92, 294). However, at a higher MOI, viral titers of ICP0 mutants show no defect compared to wild-

type virus, suggesting that there is a threshold input multiplicity above which the mutant replicates normally (94, 96, 294).

In vivo, ICP0 mutants show impaired replication and poor reactivation from latency (38, 55). Van Sant et al. reported that mutation of a single amino acid within the HSV-1 ICP0 abrogated neurovirulence in mice following peripheral inoculation and reduced neurovirulence following direct inoculation into the brain (343). Thus, it appears that in addition to activating the expression of viral proteins, degrading key host cell proteins, and counteracting the host interferon response, ICP0 may play a role in facilitating viral entry into the CNS as well.

(b) <u>US7 and US8 - Glycoproteins E and I</u>

(c) Glycoproteins E and I (gE, gI) are encoded by the US8 and US7 genes, respectively. These genes are conserved in all alpha-herpesviruses studied to date (10, 69, 197, 231, 276) These two proteins form a heterooligomer that facilitates cell-to-cell spread of the virus (70).

The HSV gE/gI complex appears to function primarily in polarized cells (epithelial cells and neurons) which form cell junctions, and not in non-polarized cells that form less extensive cell junctions (69). The cytoplasmic domain of the gE/gI complex contains tyrosine and dileucine motifs as well as acidic amino acid clusters that are phosphorylated (5, 80, 330). Similar motifs have been shown to promote endocytosis of cellular proteins and their accumulation into compartments of the trans-Golgi network (23). In HSV-1, the gE/gI complex also serves as an F_c receptor for IgG (165). The complex may actually serve to protect the virus and/or infected cell by causing IgG

aggregation or by reducing the ability of complement to bind cell or virus associated IgG (1, 71). HSV-1 gE deletion mutants showed decreased neurovirulence following intracerebral inoculation (247) and reduced neuroinvasiveness after primary infection (10).

The PRV gE/gI complex is required for spread within the CNS of pigs (180, 241). In addition, gE, and to a lesser extent gI, facilitate transport of the virus through the CNS of rats (40, 41). It is believed that gE and gI play separate roles in determining virulence, as a gI deletion mutant is more virulent than a gE deletion mutant in piglets (160). Lethal encephalitis in piglets resulting from PRV infection appears to be strictly dependent on the gE/gI complex (88). PRV gE deletion mutants fail to infect secondary neurons and show decreased neurovirulence in pigs (233). While a reduction in cell-to-cell spread in neurons caused by a nonfunctional gE/gI complex is certainly one way in which virulence is affected, there seem to be other virulence mechanisms mediated by gE/gI.

The classical PRV vaccine strain Bartha lacks both gE and gI along with several other proteins and yet is able to spread through the CNS after infection in the stomach musculature of rats (278). In fact, infection with PRV Bartha was shown to produce more infected neurons than did wild-type PRV infection. However, despite massive infection, the rodents remained symptom free and lived longer than animals infected with wild type PRV. PRV gE and gI null mutants have two phenotypes in a rodent eye infection model: restricted neurotropism and reduced virulence. One study determined that these two phenotypes reflect separate functions of the gE protein (332). While PRV neurovirulence required the carboxy-terminal domain of gE, this domain was not required

for viral spread in the rat brain. This suggests that gE may be an intrinsic virulence factor, instead of increasing virulence by promoting viral spread. This idea is corroborated by the fact that the PRV gE C-terminal domain can be phosphorylated in infected cells, possibly as part of a signaling process, and that mutations in this domain may determine a non-virulent phenotype due to loss of signaling via the gE cytoplasmic sequence (331). Further, PRV is rapidly internalized after expression on the cell surface and deletion of the gE C-terminal domain causes a defect in its internalization (331). If gE were to bind a ligand, such as IFN or TNF, that stimulated its phosphorylation and internalization in the bound state, this may elicit a pathogenic response in the infected cell that would be absent if internalization did not occur.

As in HSV, the cytoplasmic domain of the PRV gE/gI complex contains tyrosine and dileucine motifs as well as acidic amino acid clusters that have been shown to promote endocytosis of the proteins and their accumulation into compartments of the trans-Golgi network (23, 80, 330). Disruption of gI by the deletion of Val125 and Cys126 in PRV results in decreased viral replication in the oropharyngeal mucosa and no localization of the virus to the CNS (161). In addition, gI mutants replicate to normal titers at the site of inoculation in piglets, but virus can not be recovered from the CNS (180). This suggests that gI mutants are able to enter neural cells in the nasal or pharyngeal mucosa and are transported to and replicate within the trigeminal ganglion, but are then unable to spread further into the CNS.

A study of the viral genetics that influence BHV neuroinvasiveness and neurovirulence in rabbits examined differences in gE from BHV-1 and BHV-5. The

ectodomain of the neurovirulent BHV-5 gE contains a glycine rich region not present in BHV-1 and an acidic domain of the cytoplasmic tail of BHV-5 gE is longer than the acidic domain in BHV-1 gE (50). One sub-strain of BHV-1 (BHV-1.1) never invades the CNS although it encodes gE and gI homologues. However, this sub-strain is far less virulent when the gene that encodes gE is altered or missing (169). Aligned BHV-1 and BHV-5 gE amino acid sequences show 72% identity and 77% similarity (50).

(d) US9

Although the HSV-1 US9 protein product has been well characterized in the literature, a definitive function has yet to be assigned. The high degree of conservation of US9 in alpha-herpesviruses coupled with its non-essential nature *in vitro* suggests that it may play a role in either neurotropism or neuropathogenicity. The only alpha-herpesviruses sequenced to date that do not contain a US9 gene are Marek's disease virus (35) and a turkey herpesvirus (369).

In HSV-1 the US9 protein is ubiquitinated prior to incorporation into virions. Following infection, US9 associates with proteasomes in infected cells. Since the ubiquitinated US9 remains stable, it has been suggested that one of its functions in HSV-1 is to disturb host cell ubiquitin-mediated protein degradation (28).

Unlike the HSV-1 homologue which associates with nucleocapsids in the nuclei of infected cells (111), PRV US9 localizes to the secretory pathway, predominately the Golgi apparatus, within infected cells (30). US9 deletion mutants show restricted spread and decreased virulence in the rodent nervous system after intravitreal or intracerebral

inoculation (31). Two important motifs have been identified in the PRV US9: an acidic cluster containing tyrosine residues and casein kinase II phosphorylation sites, and a dileucine endocytosis signal. *In vitro* mutational analysis showed that deletion of the acidic domain affects cellular localization while mutation of the dileucine motif reduces the rate of US9 internalization into host cells (33). The US9-mediated transneuronal spread *in vivo* is dependent on two highly conserved tyrosine residues while the rate of viral spread is dependent on the phosphorylation status of two conserved serine residues (32).

One study suggested a unique function for the US9 protein in the localization of most, if not all, viral membrane proteins to axons (334). Cultures of rat sympathetic neurons were infected with a US9 deletion mutant and indirect immunofluorescence microscopy used to visualize the infected cells after incubation. In the absence of US9, tegument and capsid proteins but not viral membrane proteins were detected in axons, suggesting that US9 is responsible for the localization of these proteins *in vivo*.

The neurovirulent BHV-5 US9 has 77% identity to the neurotropic, but non-neurovirulent BHV-1 US9, but is 10 amino acids shorter. The mature BHV-1 US9 is considerably smaller in size at 18-20 kDa compared to the mature 30-32 kDa BHV-5 US9. *In vitro*, BHV-1 US9 is expressed at 3 hours PI, whereas BHV-5 US9 is not expressed until 6 hours PI (53). Using an intranasal inoculation route in rabbits, a BHV-5 US9-null mutant was avirulent and failed to invade the CNS (52). Despite the difference in size and expression kinetics, the BHV-1 US9 conferred increased neurovirulence and neuroinvasiveness to a BHV-5 US9-deleted virus in rabbits (53).

(e) UL23 - Thymidine Kinase

HSV UL23 codes for virus-specific thymidine kinase (TK) activity. The TK gene is an early expression gene and is responsible for the adenosine triphosphate (ATP)-dependent phosphorylation of thymidine for its incorporation into viral DNA. HSV-1 and HSV-2 TK deletion mutants have been shown to be 7.5-fold and 40-fold less virulent, respectively, following intra-cerebral or peripheral injection into mice (108). Coupled with information from a previous study showing that TK deficient mutants replicated efficiently in actively dividing cell cultures but poorly in serum-starved cells (163), these findings support the theory that the ability of the virus to induce TK may favor its replication in certain cell types, particularly epidermal cells and cells of the CNS, that have low levels of thymidilate metabolism.

In one study, a single nucleotide mutation which produced an amino acid change (Ser182 → Asn182) near the putative nucleoside-binding site resulted in TK activity that was approximately 1% of wild type TK (325). However, the mutant was still highly pathogenic in mice. This mutation was located in a highly conserved region that shows 97% homology between HSV-1 and HSV-2. The authors hypothesized that the amino acid change increased the hydrophilicity of the peptide thereby causing a conformational change in the nucleoside binding site leading to a reduction of the phosphorylation activity of the viral TK. A second study of acyclovir-resistant HSV-2 mutants showed that a single amino acid change (Glu105 → Pro105) in the N-terminus of the TK protein also resulted in decreased TK enzyme activity, but the mutants were still fully

neurovirulent in mice (45). These results suggest that there may be more than one mechanism by which the TK gene affects virulence.

In PRV, as in other alpha-herpesviruses, the TK is a major determinant of neurovirulence (326). PRV TK deletion mutants were unable to replicate in mouse L TK^- cells and were avirulent and unable to cause seroconversion in rabbits after subcutaneous inoculation, in mice after intraperitoneal inoculation, and in chickens after intracranial inoculation (107).

(f) UL27 - Glycoprotein B

Glycoprotein B (gB), the protein product of the HSV UL27 gene, has homologues in all herpesviruses identified to date (263, 287). The cytoplasmic C-terminus of these proteins exhibits a high degree of divergence even among closely related alpha-herpesviruses (74, 76, 202).

HSV gB has several functions which are important *in vivo*: interaction with heparan sulfate on the host target cell (361), participation in fusion of the virion envelope with host cell membranes during penetration (141), and involvement in direct cell-to-cell spread of virus (271). As a viral surface glycoprotein, gB is also immunogenic, challenging both humoral and cellular host immune responses. A cluster of four mutations in the N-terminus of the HSV-1 gB resulted in the loss of four proline residues in nonpathogenic HSZP and KOS strains as compared to the pathogenic ANGpath and 17 strains (186). The decreased pathogenicity of HSZP and KOS was attributed to an

increased immunogenicity of the gB protein from these apathogenic strains compared to the virulent HSV strains (186).

Some mutations in the HSV gB ORF have been shown to affect the formation of syncytia (143), alter the clinicopathologic characteristics of the virus following inoculation into peripheral tissues (86), and to change the rate of viral entry into cultured cells (37). The hydrophilic region of the HSV-1 gB C-terminus is essential for its biological activity and mutations in this region decrease infectivity of the virus (347). However, one study of the HSV-1 gB indicated that loss of the 41 C-terminal amino acids did not affect the function of gB in cell culture (155). An additional study of HSV-1 gB revealed an amino acid difference between a non-neuroinvasive HSV-1 strain (Val523) and a neuroinvasive/neurovirulent strain (Ala523) (366). Recombinant apathogenic viruses which contained gB coding sequence from the neurovirulent strain demonstrated increased neuroinvasiveness in mice. These results suggest that the gB protein plays a role in determining the neuroinvasive phenotype of HSV.

The PRV gB is essential for both virus entry and direct cell-to-cell spread. Evasion of the host immune system is a critical factor for PRV survival and this is accomplished in part by the ability of the virus to spread directly from cell-to-cell. As a consequence of the inability to spread cell-to-cell, PRV gB null mutants exhibit decreased infectivity (252).

PRV gB also mediates a second mechanism important to host immune system evasion: antibody-induced internalization of viral cell surface glycoproteins into infected cells (103, 104). This function has been more specifically mapped to the tyrosine-based

motif in the cytoplasmic tail of gB (102) which associates with clathrin via an adaptor molecule as an initial step in the formation of an endocytotic vesicle (182). PRV gB deletion mutants show an 80% reduction in antibody-induced endocytosis (103).

Replacing the neurovirulent PRV gB with the homologous BHV-1 glycoprotein resulted in an altered neurotropism and increased neurovirulence in piglets (121). This was an unexpected result as BHV-1 is considered non-neurovirulent. These results indicate that similar to HSV and PRV, the BHV gB is also involved in neurotropism and neurovirulence.

(g) UL41 - Virion Host Shutoff Protein

The virion host shutoff protein (*vhs*) is the product of the HSV UL41 gene. Approximately 200 copies of this 58-kDa phosphoprotein are packaged in the tegument and are therefore released into the cytoplasm immediately upon infection. Once released, *vhs* rapidly and non-specifically degrades cellular mRNA prior to the expression of viral proteins (106, 274) as well as accelerating the turnover rate of viral mRNA belonging to all kinetic classes (192, 273). While *vhs* is not essential for viral growth *in vitro*, deletion mutants exhibit a two- to five-fold reduction in burst size compared to wild-type virus in a mixed infection (193).

Homologues of the UL41 gene exist in other neurotropic alpha-herpesviruses such as VZV, EHV-1 and -4, and PRV (15) but are absent in beta- and gamma-herpesviruses, suggesting a role for *vhs* in neurotropism. The size of homologous UL41 proteins differ significantly due to highly divergent regions which are interspersed between four highly

conserved, functionally important domains (numbered I to IV) of the *vhs* protein (15). Site-directed mutagenesis of these domains was used to more specifically target sequences required for the functions of neuropathogenesis and protein shut-off. Researchers found that domains III and IV are required for both virulence and host protein synthesis shut-off (318). In addition, HSV-2 strain HG52 is defective in host protein synthesis shutoff due to a truncated UL41 protein product; thus, the C-terminal domain of HSV-2 UL41 appears to be essential for full function (95).

Vhs has both endo- and exo- nuclease activity (84, 85, 370). This activity helps redirect infected cells to produce viral proteins by degrading cellular mRNA. Both *in vitro* and *in vivo*, *vhs* exhibits specificity for mRNA (84, 85, 255, 296, 320, 370). Cleavage and degradation of mRNAs initiates near regions of translation initiation; thus, mRNAs which are translated by cap-dependent scanning appear to be degraded from the 5' end (85, 173). The targeting of *vhs* to regions of translation initiation appears to stem from association of *vhs* with the mammalian translation factor eIF-4H (105, 207).

The attenuation of HSV *vhs* null mutants *in vivo* is evident 24-48 hours PI (310, 311, 319). This suggests an important role for mediators of innate immunity in controlling these infections. While the role of HSV-1 *vhs* as a mechanism for viral evasion of IFN is still somewhat controversial (195, 239, 324), the role of HSV-2 *vhs* in mediating IFN resistance is more widely accepted (72, 243). HSV-2 *vhs* null mutants are restored to near wild-type virulence in IFN-α/β receptor ($IFNAR^{-/-}$) knockout mice (243). It appears that the anti-IFN effects of HSV-2 *vhs* are very broad as the viral protein appears to interfere with both IFN-β production by infected cells and the sensitivity of

HSV-2 to IFN-β (72). HSV *vhs* has also been shown to suppress the production of proinflammatory chemokines and cytokines *in vitro* (324). Thus, *vhs* is an important viral mechanism for evasion of the host innate immune response.

HSV-1 and -2 *vhs* have been shown to combine with the viral protein ICP47 to block antigen presentation by MHC I and decrease the expression of MHC II on infected cell surfaces as a consequence of its ability to block the *de novo* synthesis of host proteins (335). However, this effect can be transiently counteracted by IFN-γ, suggesting a possible role for this cytokine in the early immune response to HSV (329). In addition, *vhs* has been shown to inactivate human monocyte dendritic cells (295). Taken together, these findings suggest that *vhs* may play a role in evading both the innate and adaptive immune response to HSV infection.

Late in infection, UL41 is expressed for packaging into the tegument of newly formed progeny virions (319). The viral transcriptional activator VP16 complexes with *vhs* (306) and is responsible for modulating its nuclease activity to prevent degradation of viral mRNAs expressed as late genes (194, 306). Thus, VP16 is required late in infection to sustain viral protein synthesis by blocking *vhs*-mediated destruction of viral mRNAs (194).

While HSV-1 and HSV-2 *vhs* proteins are 87% identical (95), HSV-2 *vhs* degrades mRNA 40-fold faster and more completely than the HSV-1 *vhs* (99) due to several type-specific amino acid changes between the two (100). *In vitro*, cellular mRNAs are almost completely degraded by 6 hours post-infection (PI) by HSV-1 and as quickly as 2 hours PI by HSV-2 (144). However, the kinetics of *vhs* do not necessarily

directly correlate with virulence of the virus as replacement of the HSV-1 UL41 with the more efficient HSV-2 UL41 did not increase the virulence of the recombinant (310).

Both HSV-1 and -2 strains that carry mutations in the UL41 gene have been identified on the basis of their inability to degrade host cell mRNA coincident with an aberrant accumulation of IE viral transcripts (192, 273, 320). HSV-2 mutants lacking the *vhs* protein grow to significantly lower titers in mouse corneas, trigeminal ganglia, vaginas, dorsal root ganglia, spinal cords, and brains, with a corresponding decrease in their ability to induce disease (311). HSV-1 *vhs*-deficient mutants display attenuated virulence in mice and are defective for the establishment and reactivation of latent infections (12, 319). The findings of these studies strongly support the idea that *vhs* is critical for efficient *in vivo* replication of HSV-2 and may represent an actual viral determinant of neurovirulence.

The PRV UL41 gene is homologous to the HSV UL41 coding sequence. The PRV *vhs* is a 365 amino acid protein with a predicted molecular weight of 40.1 kDa (15). In PRV-infected cells a decline in the level of cellular proteins occurs, although delayed compared to HSV-1 (200).

BHV-1 also produces a *vhs* protein during infection (146). BHV-1 *vhs* has been shown to be responsible for down-regulating expression of mRNA for MHC class I molecules and other cellular proteins (129). While a gene having a predicted amino acid sequence 91% identical to the BHV-1 *vhs* has been identified in BHV-5, its protein product has yet to be characterized (68).

(h) UL44 - Glycoprotein C

Glycoprotein C (gC) is one of the major virus attachment proteins. In most alpha-herpesviruses including HSV-1, it has been shown to mediate primary attachment of the virion to the host cell by binding to heparan sulfate proteoglycans on the cell surface (120). Further, in both HSV-1 and HSV-2, gC binds complement component C3b, thus interfering with the early host immune response. This mechanism protects HSV-infected cells from early clearance by complement-mediated lysis (17, 83, 114).

A study of the attenuated vaccine strain of VZV and of HSV-1 showed that gC plays a critical role in the virulence properties of both viruses in human skin inoculation trials, with deletion mutants producing lower infectious virus yields (235). A second study used a clinical isolate which produced a truncated HSV-1 gC as the result of an amber mutation at amino acid 280 (234). This truncation did not alter the pathogenic phenotype after intracerebral, intraperitoneal or corneal inoculation of mice compared to wild type HSV-1. As the HSV strain was isolated from a human recurrent herpetic keratitis lesion, the mutant must also be able to efficiently replicate within its natural human host.

An HSV-2 mutant incapable of producing gC caused local inflammation followed by lethal infection of the CNS after intravaginal inoculation of BALB/cJ mice and intracerebral inoculation of DBA-2 mice leading to the conclusion that deletion of HSV-2 gC does not affect the neuroinvasiveness or neurovirulence of the virus (166).

Initial contact between PRV and the host cell is made between gC and heparan sulfate proteoglycans on the cell surface (232). Previously, it was thought that unlike HSV-1 which can also bind heparan sulfate with gB (141), the PRV gC was the only viral protein capable of mediating infection of cells in a heparan sulfate-dependent manner (171). However, receptor binding activity has also been shown for PRV glycoprotein D (gD) and, in gC deletion mutants, viral attachment can occur via gD (167). Even so, a PRV gC/gD double deletion mutant was infectious *in vitro*, although with a marked reduction in replication efficiency, suggesting the presence of additional attachment proteins (172).

Although the BHV-1 and -5 gC proteins are 75% identical, the N-terminal third of the proteins differ significantly (68) and variability in the heparin binding sites result in the two viruses exhibiting divergent cell surface binding phenotypes (199). In BHV-5, gC affects neurotropism and is important for high levels of virus replication and full expression of virulence in the rabbit CNS (51). In a murine vaginal infection model, a BHV-1 gC deletion mutant grew to lower titers at the site of primary infection and produced a less severe disease (208).

Additional Determinants of Neurovirulence

In addition to the action of individual viral proteins, the interaction of numerous viral and host factors determine the clinicopathogenesis of a viral infection. Besides viral genes which exist to enhance replication and virulence, both the viral dose and route of inoculation contribute to the final outcome of viral infection (29, 351, 368).

Host factors which affect the outcome of viral infection include viral receptors on various cells or tissues, and both innate and adaptive immune responses to the virus (20, 90, 195, 274, 335). Lopez was the first to put forth the idea that certain hosts may be genetically resistance to HSV infection when he showed that C57BL/6 mice were more resistant to HSV-1 infection than other inbred strains of mice (205). It was also shown that host factors play an important role in the establishment of latency in mice inoculated with HSV-1 (174). Later, it was shown that the although HSV-1 was able to enter, replicate, and spread with equal efficiency in different strains of mice, C57BL/6 mice were more resistant to HSV-1 pathogenesis (134). Host gender can also be a contributing factor in herpesvirus infections. Multiple studies have suggested that hormones play a role in HSV-2 pathogenesis by altering host susceptibility as well as the local immune response to HSV-2 infection (124, 175). Finally, several studies have shown that innate resistance to HSV infection is positively correlated with early, efficient, IFN production (36, 322).

In summary, the sum of the interactions between a given virus and an individual host defines the virulence of a virus within that particular host. This implies that caution must be exercised in identifying any particular viral gene as a definitive, universal "virulence" gene without defining the pathogenicity of the virus within the context of multiple host species.

Article II. Simian Alpha-Herpesviruses

The close genetic, anatomic, and physiologic relationship between humans and non-human primates provides not only an excellent model system to experimentally study

human disease and therapies, but also requires investigations into viruses which are endemic in these animals that may pose a zoonotic and/or cross-species threat. The zoonotic potential of primate viruses was first recognized in 1932 when a researcher died of progressive encephalomyelitis 15 days after being bitten on the hand by a healthy rhesus macaque (119, 292). Two separate groups characterized the etiological agent of the infection as an alpha-herpesvirus which is now known as *Cercopithecine herpesvirus* 1 (monkey B virus; BV) (119, 292).

Cercopithecine herpesvirus 1

BV was the first simian herpesvirus to be identified and today remains the most well-studied primate alpha-herpesvirus. The clinical manifestations of BV in its natural host, Asian macaque monkeys (*Macaca* spp.), closely resemble HSV infection in humans. Similar to other alpha-herpesviruses, serious disease as a result of BV in macaques is an exception to the benign infections more commonly observed. Seroprevalence of BV antibodies in captive, adult, macaques ranges from 73-100% (256, 257). Animals can become infected at an early age via contact with an infectious animal; however, more common is infection coincident with sexual activity (373).

Similar to HSV, primary BV infection occurs in the epithelium (352). Latency is established in the local sensory ganglia similar to HSV infections in humans (352, 373). Latent virus has been isolated from both the trigeminal and lumbosacral dorsal root ganglia, validating both the oral and genital routes of primary infection (372). Reactivation of latent BV is associated with stress, hormonal fluctuations associated with breeding/birthing, or illness (352, 373).

In contrast to the typical herpetic infections observed when BV infects macaque species, BV infections in non-macaque primates including humans produce severe, often fatal encephalomyelitis. Although only approximately 40 cases of human BV infection have been documented, without treatment the fatality rate is ≥ 70% and permanent neurologic damage is common in survivors (9, 151, 154, 256, 257, 352). Three antiviral agents are currently available for post-exposure prophylaxis of BV infections: acyclovir, valacyclovir, and famciclovir (56). Treatment with these antiviral medications may decrease the fatality rate, but only rapid diagnosis and immediate initiation of therapy can help control entry and spread of the virus in the CNS and limit neurologic damage.

In addition to humans, naturally-occurring, cross-species BV infection of cebus monkeys (59), patas monkeys (204, 357), colobus monkeys (204), Debrazza's monkeys (328), and marmosets (328) produces a fatal CNS infection. Recently, asymptomatic BV infections have been reported in capuchin monkeys after the animals were housed in the same room as BV-positive macaques (59). These animals were deemed to be positive for BV based on PCR/DNA sequencing and serology; however, these results do not definitively exclude the possibility that the virus was another closely related alpha-herpesvirus and not BV.

Herpesvirus saimiri 1

Herpesvirus saimiri 1 (HVS-1) is an alpha-herpesvirus that infects squirrel monkeys in a manner similar to human HSV and BV in macaques (61, 148, 181). Similar to BV, HVS-1 is capable of producing severe disease when transmitted to species other than the non-natural host. This includes a single report of human infection associated with contact

with squirrel monkeys. After developing non-fatal encephalitis, a psychologist reported having had contact with squirrel monkeys. Although virus isolation was never attempted, the patient did develop a rising antibody titer to HVS-1 (299).

HVS-1 was first isolated simultaneously from two non-natural host species, marmosets (149, 150) and tamarins (230), after the virus produced fatal infections in both species. HVS-1 has also been isolated from owl monkeys following fatal infection (156, 228). The severity of disease is consistent with these species representing aberrant hosts for the virus. Within these non-natural hosts, HVS-1 produced severe, disseminated, infections which affected numerous organ systems including the nervous system (149, 150, 237). In contrast, HVS-1 has been isolated from an apparently healthy tamarin, suggesting that these animals can survive infection and carry the virus latently (242). Thus, HVS-1 is very similar to BV in its propensity for producing severe, often fatal, cross-species infections.

Cercopithecine herpesvirus 2

Cercopithecine herpesvirus 2 (simian agent 8; SA8) was identified in 1958 following isolation of the virus from its natural host, an African green monkey (212). Interestingly, the initial 1958 isolate remains the only confirmed isolate of SA8 in existence. While SA8 was initially shown to be neurovirulent in mice (211, 212), subsequent studies have shown it to be decreasingly pathogenic in the mouse model (75, 279). Further, there have been no reported cases of zoonotic or cross-species SA8 infection. Recently the complete SA8 genome was sequenced (340). The overall homology at the DNA level was 83.3% between SA8 and B virus (264, 340).

(a) *Cercopithecine herpesvirus* 16

Cercopithecine herpesvirus 16 (*Herpesvirus papio* 2; HVP2) is an alpha-herpesvirus of baboons. Sequencing of the full 156,487 bp HVP2nv genome recently demonstrated an overall G+C content of 76.5% (339). Consistent with the proposed co-speciation of herpesviruses and their hosts, the closest genetic relative of HVP2 is SA8 (339). All HVP2 ORFs are colinear with and highly homologous to the corresponding ORFs in SA8, BV, and HSV (18, 264, 339).

The biology of HVP2 in baboons is typical of alpha-herpesviruses: primary infection occurs at the epithelial surface and latent infection is then established in the PNS that can subsequently reactivate, either asymptomatically or with recurrent lesions. Baboons acquire HVP2 most commonly as an oral infection during infancy/childhood or as a genital infection after becoming sexually mature (79, 261). A 2004 study of captive baboons in a breeding colony determined that approximately 60% of baboons were seropositive for HVP2 before reaching sexual maturity (approximately 3 years of age) (261). In addition, while virus was isolated from the trigeminal ganglia of 3/6 apparently healthy young baboons, no virus was isolated from the sacral dorsal root ganglia in the same study (170), thus supporting oral vs. genital acquisition of the virus in these very young animals. In either case, by adulthood > 95% of captive-born and 90% of wild-caught baboons are seropositive for the virus (78, 261).

Typical of herpesviruses, HVP2 demonstrates co-adaptation with its natural baboon host so that serious disease as a result of infection is relatively rare (226). When it does occur, severe HVP2 disease most often manifests as a fatal, disseminated infection

of neonates. Experimental infections using intratracheal and intravenous inoculations produced fatal cases of bronchopneumonia and septicemia, respectively, in neonates while slightly older baboons exhibited no clinical signs of infection (27, 81, 82). HVP2 was implicated as the etiological agent in two cases of pneumonia and septicemia in Gelada baboons which were found dead 24 hours after birth (253). Although virus was never isolated from these cases, histopathological evidence indicated the presence of a herpesvirus within the infected tissues. Recently, a naturally occurring, fatal case of HVP2 pneumonia was confirmed in a baboon infant destined for a specific-pathogen free (SPF) colony (359).

In 1988 Levin et al. reported an HVP2 epizootic in a captive population in a breeding facility (198). Consistent with what is known about alpha-herpesviruses within their natural host, oral lesions were most common in juvenile baboons with genital lesions occurring primarily in sexually mature animals. Particularly interesting was the severity of acute clinical signs and the consequent complications associated with this outbreak (198, 216). Several of the baboons with oral lesions developed very severe necrotic gingivitis and two males died as a result of severe lesions on the tongue. Vaginal obstruction and/or stenosis were the most common complications observed in older females and several of these animals either required surgery to restore their ability to breed or were deemed to have chronic herpes unresponsive to treatment and were culled from the colony after a fourth recurrence. The high rate of recurrent infections with visible, severe lesions in this epizootic outbreak was remarkable. It was suggested that the outdoor housing of these animals may have contributed to unsanitary conditions

which may have resulted in secondary bacterial infections that exacerbated the clinical disease.

HVP2 has received very little attention as a zoonotic agent in the past due to the generally innocuous clinical manifestations in baboons and the lack of reported cases of interspecies transmission from baboons to humans. However, while HVP2 is very similar genetically and phenotypically to the apathogenic SA8, it is also very closely related genetically to the neurovirulent BV (75, 77). The continued and increasing use of baboons as research models, as well as advancements in xenogenic organ transplants using baboon donors, necessitates a clearer picture of the virulence capabilities of the virus.

During the production of immune sera in mice, rapid onset death was observed in female BALB/c mice inoculated intraperitoneally with one isolate of HVP2 (77). In subsequent investigations comparing the pathogenicity of SA8 and HVP2 to HSV-1 in mice, two strains of HVP2 were found to readily invade the CNS following intramuscular inoculation, resulting in infections as severe as those produced by the most virulent strains of BV (279). When additional strains of the HVP2 were tested in female BALB/c mice, some were similarly neurovirulent while others produced neither clinical signs of disease nor histopathological signs of infection (283).

To confirm these results, mice were inoculated intramuscularly with high doses of numerous HVP2 isolates obtained from different baboon subspecies and primate centers over a span of some 20-30 years (283). Some of the HVP2 isolates (6/13) caused paralysis and death in the mice, while other HVP2 isolates (7/13) produced no clinical

signs of disease. The apathogenic HVP2 isolates (HVP2ap) induced only low levels of serum anti-viral IgG relative to levels observed in sera from mice infected with the neurovirulent isolates of HVP2 (HVP2nv). Histological examination of tissues from mice inoculated with HVP2nv isolates showed extensive neural tissue destruction, while mice infected with HVP2ap isolates showed no lesions in either the PNS or CNS. Histological and immunopathological evidence of viral infection at the site of inoculation was marked in HVP2nv-infected mice but absent in mice infected with HVP2ap.

It was surprising that with the relatively large number of HVP2 isolates tested; only the two dichotomous pathogenic phenotypes were observed in mice. This is in contrast to strains of BV which exhibit a wide spectrum of pathogenic phenotypes that span the range from completely apathogenic to extremely neurovirulent (280).

Phylogenetic analyses of both coding and intergenic regions (US4-6) of the HVP2 genome separated isolates into two distinct clades that correlated exactly with the two *in vivo* virulence phenotypes. This same region of the BV genome has been shown to differentiate BV isolates from different macaque species (254, 307). Differences located in non-coding sequence consist of extensive insertions/deletions between different BV strains. In contrast, differences in the same region between isolates of HVP2 were relatively minor, most being single nucleotide changes and small insertions/deletions. In addition, sequencing of this region did not support the existence of HVP2 genotypes specific to individual baboon subspecies (283).

Despite the apparent inability of HVP2ap to cause disease in mice, there was no significant difference in the *in vitro* replication, plaque size, or CPE morphology of

HVP2nv vs. HVP2ap isolates in either Vero or murine L cells (283). The results of this study identified two subtypes of HVP2 that are very closely related genetically, but which differ dramatically in their ability to cause disease in a mouse model.

Significance of HVP2 Research

The dichotomous pathogenicity of HVP2 isolates within a single, genetically identical host species (i.e. BALB/c mice) supports the use of an HVP2 model system for studying the viral genetic determinants of cross-species or zoonotic pathogenicity. A close genetic, antigenic, and biological relationship has been established between HVP2 and BV as well as with other neurotropic alpha-herpesviruses so that valuable insight into these viruses can likely be extrapolated from HVP2 investigations. In addition, comparisons of viral pathogenicity in the natural vs. non-natural host would be useful in determining viral factors responsible for promoting neurovirulence in cross-species or zoonotic infections. Experimental manipulation of the natural baboon host is plausible in contrast to HSV research involving human subjects or BV studies involving macaques which must be conducted under BSL-4 conditions. Thus, while HVP2 has been historically overlooked due to the apparent lack of zoonotic potential, it may prove to be an invaluable tool for increasing knowledge of how more relevant alpha-herpesviruses cause disease.

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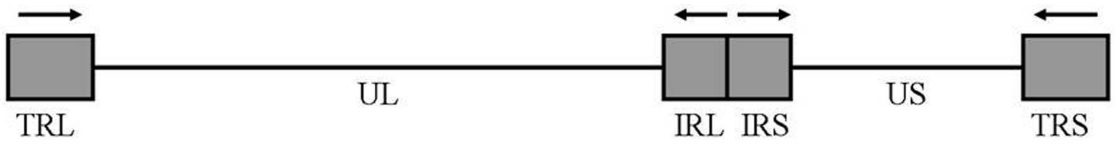

Figure 2.1 - Genome organization of the prototypic alpha-herpesvirus HSV-1. A typical alpha-herpesvirus genome contains unique sequence (UL and US) flanked by repeat regions which are either terminal (TRL and TRS) or internal (IRL and IRS) to the unique sequence. Arrows indicate the orientation of each repeat sequence.

CHAPTER III

DNA SEQUENCE ANALYSIS OF POSSIBLE VIRAL DETERMINANTS OF NEUROVIRULENCE IN THE *Herpesvirus papio* 2 GENOME

Introduction

In its baboon natural host, *Cercopithecine herpesvirus* 16 (*Herpesvirus papio* 2; HVP2) behaves similarly to herpes simplex virus type 1 (HSV-1) of humans and *Cercopithecine herpesvirus* 1 (monkey B virus; BV) of macaques. Primary infection occurs at an epithelial surface. Latent infection is then established in the peripheral nervous system (PNS) that can be reactivated, either asymptomatically or with recurrent lesions. In their natural hosts, these viruses only rarely cause serious neurological disease, and most such cases occur in infants or immune compromised individuals. In contrast, these viruses can produce severe infections of the central nervous system (CNS) when they infect non-natural host species (147).

A number of HVP2 isolates (HVP2nv) collected from different sub-species of baboons housed at various primate centers proved to be extremely neurovirulent in a murine intra-muscular inoculation model system (114, 118). In fact, these HVP2nv isolates produce CNS infections which parallel the most severe infections seen in mice following inoculation with BV. A second group of HVP2 isolates (HVP2ap) are

apathogenic in mice, failing to replicate efficiently at the primary site of inoculation and to invade the CNS (119).

DNA sequencing and phylogenetic analysis of a 1.1 Kbp region between US4 and US6 divides HVP2 isolates into two distinct genetic clades that correlate completely with their neurovirulent or apathogenic phenotype in mice (119). While this genomic region also divides BV isolates into genotypes based on the macaque species from which the virus was isolated (130), no such host species partition is evident from analysis of this same region in HVP2 isolates (119). In short, there is more genetic variation between genotypes of BV than between HVP2 subtypes. This finding led to the hypothesis that more significant genetic differences exist in other areas of the HVP2 genome. Restriction fragment length polymorphism of numerous HVP2 isolates using several different restriction endonuclease enzymes supports this hypothesis (119).

Herpesviruses have double-stranded DNA genomes of considerable size and complexity. The typical herpesvirus produces more than 30 structural proteins and has more than 70 viral genes (122). Because of this large genome size, herpesviruses produce proteins not only to facilitate viral replication, but also proteins for enhancing the infection process within a host organism. The alpha-herpesviruses contain genes which function to allow these viruses to both productively infect and maintain latency within the host nervous system. Numerous viral determinants of neuroinvasiveness and neurovirulence have been identified by characterizing interactions that occur within the infected host following primary infection at epithelial surfaces. Theoretically any gene which impairs viral replication will negatively affect virulence such that all essential

genes could be considered virulence determinants (37, 85). However, numerous non-essential alpha-herpesvirus genes have been shown to be required for maximum neuroinvasiveness (75, 153) and neurovirulence (9, 22, 110, 138, 140) in various animal models.

Based on different genes that have been shown to affect neurovirulence of HSV and other well-characterized alpha-herpesviruses such as pseudorabies virus (PRV) of pigs, a number of HVP2 genes were identified that might potentially determine the differential neuropathogenicity of the two HVP2 subtypes in mice. The goal of the experiments presented here was to perform comparative sequencing of these target genes from representative HVP2nv and HVP2ap isolates and determine if there are DNA sequence differences that correlate with the neurovirulence phenotype of the two HVP2 subtypes. DNA sequences from six HVP2 isolates (3 of each subtype) were compared at both the nucleotide and amino acid levels in an attempt to identify major coding differences (i.e. premature stop codons, frameshifts, etc.) which might explain the pathogenic disparity between the two HVP2 subtypes. In the absence of any major coding differences, HVP2-subtype-specific amino acid changes were identified and examined to determine how these amino acid changes might affect the function of the viral proteins.

Materials and Methods

Cells and Viruses

Vero cells were originally obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM containing 2% FBS. Primary mouse dermal fibroblast (PMDF) cell cultures were prepared and cultured as described (117).

The origins of all HVP2 isolates are detailed in Table 3.1 and have been previously described (43-45, 119).

DNA Sequencing and Analysis

Polymerase chain reaction (PCR) was used to amplify coding sequence for most genes of interest from three HVP2nv isolates (OU1-76, X313 and A189164) and three HVP2ap isolates (OU2-5, A951, and OU4-8). Sodium iodide gradient purified viral DNA was used as PCR template (130) and PCR conditions were as previously described (119, 130, 141) with an extension time of 1 minute/Kbp for each reaction. All PCR products were purified prior to sequencing using the Wizard PCR Preps (Promega, Madison, WI).

Sequencing of regions that proved problematic by PCR was completed using the primer walking method. Sufficient flanking sequence was necessary to identify restriction enzyme sites that would cut the region of interest from HVP2 genomic DNA. Universal plasmid primers were used for end sequencing and to obtain DNA sequence for

design of internal sequencing primers. Vector NTI Suite 8.0 (InforMax, Inc., Frederick, MD) software was used to assemble and align all DNA and protein sequences.

Acyclovir Sensitivity Assay

Confluent monolayers of Vero cells in 24-well trays were infected with two strains of HVP2ap (OU2-5 and A951) or two strains of HVP2nv (OU1-76 and X313) at an MOI of 1.0 PFU/cell. All viral dilutions were made using 2% FBS maintenance medium. A stock solution of acyclovir (ACV) (Acycloguanosine, Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/ml was prepared using dimethyl sulfoxide (DMSO) as a solvent. Once infected, cells were incubated at 37 °C. After one hour the inoculum was removed, cells were washed twice with sterile phosphate-buffered saline (PBS), 500 µl of maintenance media containing ACV at concentrations of 0, 2.0 or 20 µg/ml was added to the infected cells, and the trays returned to 37 °C. At 24 hours post-infection (PI), infected cells were scraped into the media and subjected to three cycles of freeze/thaw before being assayed for infectious virus using a standard plaque assay on Vero cells (119). Plaques were counted at 2 days PI.

RNA Isolation from HVP2-Infected Cells

Total infected-cell RNA was isolated using the RNeasy mini-prep Kit (Qiagen, Valencia, CA) followed by three NaOAc precipitations of the RNA sample. Final RNA sample concentrations and purity (OD_{260}:OD_{280} ratio ≥ 2.0) were determined using an Eppendorf BioPhotometer V1.26 (Westbury, NY).

Reverse Transcription and Quantitative Real-Time PCR

Total RNA was isolated at designated time points following infection of confluent PMDF cell cultures in 12-well trays with 4 or 0.4 PFU/cell HVP2nv (OU1-76 strain) or HVP2ap (OU2-5 strain). First strand cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) as directed by the manufacturer using a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA). All cDNA samples were stored at -80 °C prior to use in the quantitative real-time PCR assay. Samples with no reverse-transcriptase were prepared as negative controls to ensure the absence of viral genomic DNA in cDNA samples.

Quantitative real-time PCR was used to amplify RL2 and UL41 cDNA from HVP2nv- and HVP2ap-infected PMDF cell cultures. HVP2-specific primers and Taqman probes were designed using the PRIMEREXPRESS software (Version 2.0, Applied Biosystems, Foster City, CA, USA). To avoid HVP2-subtype-specific sequence variation, primers and probes were designed from the consensus sequence produced by alignment of the HVP2 ORFs of interest from four HVP2ap and four HVP2nv isolates. The RL2 cDNA was amplified using the primer pair F (5'-TATCTCATCGTGGGCGTGA-3') and R (5'-TCGTTGACCACCGGGAT-3') with a TaqMan® MGB probe (5'-GCCCGACGGCTCGTACAGCA-3'). HVP2 UL41 cDNA was amplified using primers F (5'-TGCGCCAACCTCTACCA-3'), R (5'-TGTCGGTCGTGTGGACGT-3') along with a TaqMan® MGB probe with sequence (5'-CCAACACCGTCGCG-3') (118). Both probes were labeled at the 5' end with FAM (6-carboxyfluorescein) and at the 3' end with a non-fluorescing quencher. All real-time

PCR reagents, disposables, and equipment were purchased from Applied Biosystems. PCR reactions were performed in a 96-well plate and each 25 µl reaction contained 1x TaqMan® Universal PCR Master Mix, 900 nM each primer, 250 µM probe, and 5.0 µl sample cDNA. Results for detection of RL2 and UL41 cDNA were normalized to copies of the 18S rRNA gene amplified by the TaqMan® Pre-Designed Assay for 18S rRNA as previously described (118).

PCR amplification and detection was performed on an ABI Prism 7000 Sequence Detection System using the following cycling conditions: 1 cycle 50 °C for 2 min, 1 cycle 95 °C for 10 min, and 40 two-step cycles of 95 °C for 15 sec and 60 °C for 1 min. All PCR reactions were carried out in triplicate with appropriate controls run on each plate including no template controls and no reverse-transcriptase controls.

For use as an RL2 standard in the PCR assay, a 700-bp segment of the HVP2 RL2 second exon was amplified by standard PCR using the forward primer HVP2-1502 5'-GACCTCCTGCTGCTCTCCCGCTAC-3' and the reverse primer HVP2-1503 5'-CGTCCAGATGAAGTCCACGGCCGT-3' (Sigma-Genosys, The Woodlands, TX). The Invitrogen (Carlsbad, CA, USA) TOPO TA Cloning® Kit was used to clone the PCR product and fidelity of the insert was confirmed by DNA sequencing. Purified plasmid DNA was isolated using the QIAGEN Plasmid Maxi Kit and quantitated by spectroscopy. The plasmid standard was used as described on the Applied Biosystems website (*www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf*). The dynamic range for detection of the HVP2 RL2 plasmid standard by the real-time PCR assay was

10^1-10^5 copies. The HVP2 plasmid standard design and validation has been previously described and shown to have a dynamic range of 10^0-10^5 copies (118).

Northern Blot Analysis

Confluent monolayers of Vero or PMDF cell cultures were infected at an MOI of 1.0 PFU/cell or mock infected and incubated at 37 °C (Vero) or 39 °C (PMDF) for 4 hours. RNA samples (15 µg) were purified as described above, resolved by formaldehyde-agarose gel electrophoresis and northern blotting performed as described (111, 112). Blots were hybridized with a ^{32}P-labeled β-actin DNA probe prepared by asymmetric PCR using Vero cell DNA. Relative β-actin mRNA levels were determined by autoradiography.

Complement Neutralization Assay

Antibody-independent complement (C') neutralization was performed by incubating 10^4 PFU of HVP2nv (OU1-76 and X313) or HVP2ap (OU2-5 and A951) with non-immune baboon serum (as a C' source) or heat-inactivated (56 °C for 30 min) non-immune baboon serum at 37 °C. After one hour, $1x10^5$ Vero cells in 150 µl of maintenance medium were added to each well of the 96-well tray containing the C'-virus mixture. At 72 hours PI the wells were examined for viral cytopathic effect (CPE). Non-immune guinea pig serum served as a positive C' control. HSV-1 strains KOS (wild-type) and MP (gC^-) were used as positive and negative viral controls, respectively.

Non-immune baboon sera were obtained from juvenile olive baboons (*Papio cynocephalus anubis*) in the specific pathogen free (SPF) program at the Oklahoma

University Health Sciences Center (OUHSC). To confirm that the sera contained no antibodies to baboon viruses, ELISA was performed as previously described (101) except that peroxidase-conjugated anti-human IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody at a 1:5000 dilution. Baboon sera were tested at a 1:100 dilution. All baboon serum samples used in these experiments were negative by ELISA for serum antibodies to 11 viruses: HVP2, *C. herpesvirus* 12 (*Herpesvirus papio* 1; HVP1), baboon cytomegalovirus, *C. herpesvirus* 17 (macaque rhadinovirus), *C. herpesvirus* 9 (simian varicella virus), simian T lymphotrophic virus, simian foamy virus, simian retrovirus, simian immunodeficiency virus, simian virus 40, and measles (104, 120).

Results and Discussion

In an attempt to identify genomic sequence differences which may account for the dichotomous pathogenic phenotypes of HVP2 subtypes in mice, ten genes were sequenced from three HVP2nv isolates and three HVP2ap isolates. These genes were chosen based on evidence for their involvement in neuroinvasiveness and/or neurovirulence in other closely related alpha-herpesviruses. The details of the comparative sequencing are summarized in Table 3.2.

RL1 (ICP34.5)

The RL1 gene which codes for infected cell protein 34.5 (ICP34.5) has been identified in HSV-1 and -2 as well as in a macropodid alpha-herpesvirus. Because the RL1 is located in the repeat region, it exists in diploid form within the HSV genome.

The RL1 gene codes for ICP34.5 which is a low-abundance, early protein (92) responsible for precluding the total shut-off of protein synthesis by the host cell in response to infection. ICP34.5 redirects the double-stranded RNA-dependent protein kinase (PKR) so that the alpha subunit of eukaryotic translation initiation factor 2 (eIF-2α) remains dephosphorylated and protein synthesis continues (71).

HSV-1 RL1 deletion mutants fail to prevent total shut-off of protein synthesis, thereby rendering the host cell unsuitable for viral replication (31). This mutation severely decreases viral yields. Loss of RL1 corresponds to a completely avirulent phenotype in experimental HSV-1 mouse and guinea pig model systems (149).

Analysis of DNA sequence from the long repeat region of HVP2nv strain OU1-76 and HVP2ap strain OU2-5 did not reveal any potential RL1 ORF. Two other research teams concluded independently that the HVP2nv isolate X313 also lacks an RL1 homologue (11, 144). Finally, the lack of an RL1 gene in HVP2 corresponds with the lack of an identifiable RL1 ORF in the genome of both BV (106) and SA8 (145).

The lack of an RL1 gene in the simian alpha-herpesviruses may be explained by examining the proposed origin of the HSV RL1 gene. The C-terminal sequence of the HSV-1 RL1 gene shows remarkable homology to the mammalian growth arrest and DNA damage (GADD34) protein and to the murine myeloid differentiation primary response (MyD116) protein (16, 30, 70). These proteins suppress cellular growth during DNA repair to preclude stress responses that could result in apoptosis. The current hypothesis is that HSV may have "borrowed" this coding region from its human host to allow the virus to block a cellular response to infection and preclude total protein synthesis

shutdown and apoptosis (70). However, the size of the repeat region is similar in the human and non-human primate viruses. Conservation of this amount of sequence suggests that it is important to these viruses in some as yet unresolved manner.

RL2 (Infected Cell Protein 0)

Infected cell protein 0 (ICP0) is encoded by the diploid RL2 gene located in the inverted repeat sequences which flank the long unique region of the herpesvirus genome. ICP0 consists of three exons and is expressed as an immediate-early protein. ICP0 in its HSV-1 form is conserved in few herpesviruses. Nevertheless, several alpha-herpesviruses including PRV (29), equine herpesvirus type-1 (137), bovine herpesvirus type-1 (150), SA8 (145), and BV (106) contain genes which are at least in part homologous to the HSV-1 ICP0.

The HSV-1 ICP0 is a multifunctional protein which plays many diverse roles in viral infection. As an immediate-early protein, ICP0 promotes transcription of viral early genes through interaction with both viral and cellular transcription factors. ICP0 is also important for counteracting the anti-viral actions of type I interferons (IFN) (46, 68, 95). Finally, ICP0 is also responsible for regulating steady-state levels of both viral and cellular proteins via its E3 ubiquitin ligase domain in conjunction with the cellular protein degradation machinery (15, 146).

In the absence of ICP0, HSV-1 is severely impaired in its ability to replicate at the site of inoculation and to establish latency in the PNS (19, 35). HSV-1 ICP0 null mutants are significantly less pathogenic in both mice and rabbits following intra-ocular

inoculation (65). Mutation of a single amino acid in the HSV-1 ICP0 made the virus non-neuroinvasive in mice following peripheral inoculation and slightly less neurovirulent following direct inoculation into the mouse brain (147).

While the predicted protein sequence of HVP2 ICP0 showed no subtype- specific amino acid changes, the ICP0 protein sequence did reveal remarkable deviations for individual HVP2 isolates. However, all splice donor/acceptor sites were conserved so that the HVP2 isolates examined appear to produce full-length ICP0 proteins. The RING finger zinc-binding motif identified in HSV ICP0 proteins (50) was also present and highly conserved among all HVP2 isolates. As expected, the DNA sequence of RL2 introns was more variable between individual isolates of HVP2 than was the RL2 coding sequence. RL2 intron sequences are primarily comprised of reiterated sequence with variable numbers of repetitions found in each HVP2 isolate.

The divergent RL2 DNA sequence between individual isolates of both HVP2 subtypes was interesting due to the importance of ICP0 during productive infection. Intuitively, it would seem that such an important viral gene would be highly conserved among individual isolates of HVP2. To determine if either quantitative or temporal differences in ICP0 expression contributed to the dichotomous pathogenicity of HVP2 subtypes in mice, real-time PCR was used to characterize RL2 mRNA expression following infection of PMDF cell cultures with either HVP2nv or HVP2ap at an MOI of 4.0 or 0.4 PFU/cell. As shown in Figure 3.1, neither the copy number of RL2 mRNA transcripts nor the kinetics of ICP0 expression differed between HVP2 subtypes at any time point sampled at an MOI of 4.0 PFU/cell. A similar pattern was observed following

infection with an MOI of 0.4 PFU/cell (data not shown). As there are numerous steps between transcription and post-translational processing that affect the actual level of protein expression, the number of transcripts does not necessarily correlate with the actual quantity of protein produced. However, while differences may exist downstream from transcription of the RL2 gene for HVP2nv vs. HVP2ap, results of the quantitative real-time PCR suggest that the two viruses produce comparable levels of RL2 mRNA at both early and late times PI.

The complexity associated with the ICP0 sequence and protein function coupled with the variant coding sequences identified in HVP2 isolates warrants careful consideration of HVP2 ICP0 as a neurovirulence factor as more details emerge on the biological differences that exist between HVP2 subtypes in mice.

Article III. US7 and US8 (Glycoproteins I and E)

Glycoproteins E and I (gE/gI) are encoded by the US8 and US7 genes, respectively, and are conserved in all alpha-herpesviruses examined to date (3, 32). Both gE and gI proteins are expressed with late kinetics. These two transmembrane proteins form a heterodimer that facilitates direct cell-to-cell spread of the virus (38). In addition, the gE/gI complex acts as an F_c receptor for IgG, thereby protecting the virus and/or infected cell either by causing IgG aggregation or by reducing the ability of complement to bind cell- or virus-bound IgG (1, 39, 78).

HSV-1 gE deletion mutants exhibit decreased neurovirulence following intra-cerebral inoculation (98) and reduced neuroinvasiveness after peripheral inoculation

(3). Disruption of the PRV gI results in no localization of the virus to the CNS (76) as gI facilitates transport of the virus through the CNS (24, 25, 81).

Complete ORFs for both gE and gI were identified in both subtypes of HVP2. Comparison of the gE protein sequences from HVP2 subtypes revealed only a single subtype-specific amino acid change in the signal sequence and no differences in the predicted transmembrane domain (Figure 3.2). Four amino acid changes peculiar to HVP2 subtypes were identified in a hypervariable region of the extracellular domain of gE consisting of approximately 16 amino acids. This region is only minimally conserved between HSV-1 and -2 (corresponding to the hypervariable region of HSV-1 gE). While the first 70 residues of the N-terminal region of the gE extracellular domain were highly conserved between HVP2 subtypes, the region spanning amino acids 105 -155 contained a total of ten subtype-specific amino acid differences. This region has previously been reported to be highly variable among the primate viruses, with the exception of a small, highly conserved stretch of 24 residues flanked by two cysteine residues (100). This same 24 residue stretch was highly conserved between HVP2 subtypes, as were the three N-linked glycosylation sites (N-X-T/S) in this region. Sequence nearer the C-terminus of the extracellular domain of gE was also fairly well conserved between the two HVP2 subtypes. Finally, the cytoplasmic tail of gE contained only three conservative amino acid changes between HVP2 subtypes.

As with gE, the HVP2 gI signal sequence and transmembrane domains were very conserved, showing no subtype-specific variations (Figure 3.2). Three of the 12 subtype-specific amino acid changes were located in the cytoplasmic tail region and

the remainder of the differences occurred in the N-terminal and central regions of the extracellular domain.

HSV research characterized sequences in both gE and gI which are responsible for forming the gE:gI complex and the gE:gI:IgG complex (4, 5, 40). Previous studies indicated that these regions are not highly conserved among the primate alpha-herpesviruses (100). In HSV-1, mutation of individual residues between amino acids 235 and 380 identified the regions of gE which are important for IgG monomer binding while linker insertion mutagenesis at amino acids 235 and 264 resulted in the loss of the gE/gI complex in infected cells (4). Further, mutation of residues 210 (outside of the Fc binding domain) and 380 (inside the Fc binding domain) of gE both reduced the ability of HSV-1 to enter the sensory ganglia and initiate zosteriform spread in mice (126). Comparison of the HVP2 gE proteins revealed a single subtype-specific non-conservative amino acid difference in the region which aligned with HSV-1 residues 235-380; however, this variance is much less than the difference between HSV-1 and -2 in the same region.

In HSV-1 gI, residues 128-145 were shown to be necessary for monomeric IgG binding (5). There was a single conservative subtype-specific amino acid difference in the region of HVP2 gI corresponding to HSV-1 residues 128-145.

The accumulation of ten amino acid differences within a 50 residue stretch in the N-terminus of the gE extracellular domain may warrant further examination as more details on the *in vivo* biology of the HVP2 subtypes emerge. While this region is not highly conserved between HSV-1 and -2 or between the human and simian viruses,

it is intriguing that subtype-specific amino acid differences outnumber strain-specific differences 5:1 within such a small section of the protein.

US9

The US9 gene encodes a small type II membrane protein found in the virion tegument which is highly conserved in all alpha-herpesviruses sequenced to date (36, 55, 80, 86, 107, 144, 145). US9 is expressed as a late gene and associates with nucleocapsids in the nuclei of infected cells (55). In HSV-1, US9 is packaged into the tegument in a ubiquitinated form (17).

US9 has been shown to be important for both anterograde neuronal spread and neuropathogenesis in HSV, PRV and BHV-5 (17, 18, 34). An HSV-1 US9 null mutant spread normally within the mouse cornea and to the trigeminal ganglia; however, the mutant virus was unable to spread in anterograde fashion for return to the cornea (108).

Both HVP2 subtypes code for a complete US9 protein. Comparison of the US9 sequences revealed no subtype specific amino acid changes and only a single conserved strain specific change (OU1-76 S28 → G28). Both HVP2 subtypes had two conserved tyrosine residues (YY) which are highly conserved among all US9 homologues (80, 100). Further, both HVP2 subtypes contained a cluster of 6 arginine residues at positions 56-61. Similar clusters in HSV have been identified as a nuclear accumulation sequence (55). The high degree of identity between the US9 coding sequence from HVP2nv vs. HVP2ap argues against US9 contributing to the differential pathogenicity of HVP2 subtypes in mice.

US11

US11 codes for a site- and conformation-specific RNA binding protein in other alpha-herpesviruses (6, 123, 125). US11 is expressed as a true late protein and is one of the most abundant HSV proteins found in infected cells late in infection (79, 88). The HSV US11 protein overcomes the PKR-mediated block of protein synthesis in infected cells, allowing viral protein synthesis to continue (26, 96). The US11 protein is packaged into the virion tegument so that the protein is present prior to PKR activation in newly infected cells (26, 123). The US11 protein is critical for proper late viral translation rates (97). Within infected cells, the US11 protein localizes to the nucleolus (91), associates with polysomes (123), and binds to and regulates accumulation of a truncated, non-polyadenylated form of UL34 mRNA (124).

HSV US11 null mutants have not been shown to be impaired for virulence in mice (93, 99). However, in cells infected with a US11 mutant, viral protein translation rates were reduced 6- to 7-fold, and viral replication was reduced 13-fold compared to replication in cells infected with either wild-type or revertant virus (97).

The HVP2 US11 was highly conserved between viral subtypes with no subtype-specific nucleotide differences. Previous comparison demonstrated that the BV and HSV US11 polypeptides are strongly conserved in the C-terminal region of the protein which contains numerous reiterations of the sequence R-X-P (100). This region is responsible for binding RNA, localizing the protein to nucleoli, associating the US11 protein with cellular ribosomes, and inhibiting PKR activation (109, 125). Different isolates of HVP2 had variable numbers of the R-X-P reiteration, either 21 or 32. This is similar to what

has been reported for strains of HSV-1 which contain either 21 or 27 reiterations (109, 115). In HSV-1 it has been proposed that these repetitive R-X-P domains form poly-L-proline II helices which aligns the basic arginine residues on a single face of the helix (125). Interestingly, the number of R-X-P reiterations in HVP2 isolates did not parallel the viral pathogenic phenotype in mice but rather correlated with the baboon facility from which the virus was isolated. The additional 11 reiterations result from an exact duplication of the 33 nucleotides preceding the insertion, suggesting that variable numbers of reiterations may have arisen from a common mechanism such as replicative slippage (116, 136). The lack of HVP2 subtype-specific differences suggests that the US11 does not contribute to the disparate pathogenicity of HVP2 subtypes in mice.

US12 (Infected Cell Protein 47)

The alpha-herpesvirus US12 gene encodes infected cell protein 47 (ICP47) which is expressed in infected cells as an immediate early protein. To date, US12 homologues have been identified in several primate viruses including HSV-1 and -2 (60, 74), BV (100), SA8 (14), and HVP2 (12).

The HSV ICP47 binds to the cellular transporter associated with antigen processing (TAP) protein complex and inhibits translocation of peptides into the endoplasmic reticulum for loading onto nascent major histocompatibility complex (MHC) class I molecules (2, 74, 152). This interaction decreases viral antigen presentation by MHC I and subsequently attenuates CD8+ T-cell mediated recognition of virally infected cells (58, 59, 151).

One major limitation to determining the role of the ICP47 in HSV pathogenesis is the fact that the HSV and other homologous ICP47 proteins demonstrate an approximate 100-fold decrease in the binding of ICP47 to murine compared to human TAP complexes (60, 74, 142). This makes assessing the contribution of ICP47 to virulence difficult in experimental small animal models. One study utilized a recombinant HSV-1 strain which contained the murine cytomegalovirus US11 so that antigen presentation was efficiently inhibited (103). This recombinant HSV-1 strain replicated to higher titers in the CNS and induced paralysis more frequently in mice following rear footpad inoculation.

Comparison of the ICP47 protein sequences revealed no HVP2 subtype-specific differences. The US12 from HVP2nv isolate X313 has been previously sequenced and compared to HSV, BV, and SA8 (14). This comparative analysis revealed that several differences existed between the simian viruses compared to HSV-1 and -2. For instance, the active site of the HSV ICP47 protein has been mapped to the N-terminal 35 residues (62). Within this region a tryptophan residue at position three is important for interaction with the endoplasmic reticulum membrane (8). None of the simian viruses examined including both subtypes of HVP2 have a tryptophan residue in the N-terminal region. This may reflect differences in the TAP proteins between different host species. The lack of any subtype-specific amino acid differences in the HVP2 ICP47 coupled with a lack of evidence supporting the role of ICP47 in virulence makes it unlikely that US12 coding sequence accounts for the pathogenic differences between HVP2 subtypes in mice.

UL23 (Thymidine Kinase)

The UL23 gene codes for a virus-specific thymidine kinase (TK) protein in the alpha-herpesviruses. The TK is expressed as an early protein in infected cells and is responsible for ATP-dependent phosphorylation of thymidine for incorporation into viral DNA. While TK is not required for viral replication *in vitro* or *in vivo*, HSV TK null mutants replicate efficiently in active cell cultures but poorly in serum-starved cells (77).

TK^- PRV isolates exhibit a complete loss of neuropathogenicity in various small animal models (82, 128) as well as the natural pig host (52). HSV-1 and HSV-2 TK deletion mutants have been shown to be 7.5-fold and 40-fold less virulent, respectively, following intra-cerebral or peripheral injection into mice (54). However, a mutation in the HSV-1 TK coding sequence resulted in a virus with only 1% TK activity but full virulence capacity in mice compared to wild-type HSV (135). Further, a single amino acid change produced an HSV-2 virus with significantly decreased TK activity but full neurovirulence in mice (27). These results suggest that there may be more than one mechanism by which the TK gene affects virulence.

Acyclovir (ACV) is a synthetic, acyclic, purine-nucleoside analogue and is used as the standard therapy for herpesvirus infections (148). Following uptake of ACV by virus-infected cells, phosphorylation of the drug by the viral TK enzyme produces ACV-monophosphate which is subsequently converted to the active form of the drug ACV-triphosphate by host cell enzymes. The viral DNA polymerase then incorporates the tri-phosphate form of ACV into the nascent viral DNA where it causes chain termination. Since cellular TK enzymes do not efficiently phosphorylate ACV to the monophosphate

form, virus infected cells are preferentially affected by drug activity. The anti-viral efficacy of ACV is compromised for viral TK mutants that exhibit decreased TK production or altered enzyme specificity wherein ACV is not efficiently phosphorylated. Genotypic analysis of 30 ACV resistant HSV strains showed that 46.7% of the isolates had 1-2 nucleotide insertions/deletions while 53.3% of the isolates had point mutations in the TK (61). The link between loss of HSV-1 and -2 virulence and ACV sensitivity has been examined both in the natural host and in experimental small animal model systems (69, 102). While the majority of ACV resistant isolates are impaired for growth at the site of inoculation and within the PNS compared to wild-type virus (53, 54, 129, 139), some isolates which are insensitive to ACV retain full virulence in mice (67, 69).

The TK protein sequences from both HVP2 subtypes differed by a total of five residues, with three of these being non-conservative amino acid changes. One each of the non- and conservative amino acid changes between the HVP2 subtypes occur in the first 30 amino acids, a region that is not very highly conserved between the HSV-1 and -2 TK. Previously, an "active center" model for HSV-1 TK had been proposed in which three highly conserved regions participate in forming the active site of the enzyme (66). These three regions are all highly conserved between HVP2 subtypes, with no subtype-specific amino acid changes being located in any of these regions.

The ACV sensitivity of both HVP2 subtypes was assessed using a standard plaque reduction assay with ACV treatment. ACV sensitivity based on activation of the pro-drug via TK-induced phosphorylation was used as an indirect measure of the TK activity of both HVP2 subtypes. This assay was based on the assumption that phosphorylation of

ACV by HVP2 would decrease viral replication and thus the quantity of infectious virus produced.

Although there is no uniform agreement on what level of *in vitro* susceptibility indicates resistance, the 50% effective concentration [EC_{50}] for ACV sensitive strains of HSV-1 and -2 are generally defined as 1.0 and 2.0 µg/ml, respectively (28, 49). As shown in Figure 3.3, HVP2 isolates were only sensitive to ACV to 20.0 µg/ml. One exception was HVP2ap isolate A951 which was also sensitive to 2.0 µg/ml. Thus, HVP2 does not appear to efficiently phosphorylate ACV. Alternatively, if ACV is phosphorylated by HVP2, it is not efficiently incorporated into the viral DNA. Further, in the absence of any subtype-specific amino acid differences in conserved domains of the protein, it is unlikely that the HVP2 TK determines the pathogenic phenotype of these viruses in mice.

UL27 (Glycoprotein B)

The UL27 gene encodes glycoprotein B (gB) which is essential for viral replication *in vitro* (87) and important *in vivo* (113). Homologues of gB have been identified in all *Herpesviridae* subfamilies (105, 121). The strong conservation of the gB polypeptide among the alpha-herpesviruses suggests a common and important function for this protein (41). The HSV gB adsorbs to proteoglycans on cell surfaces, participates in virus-cell fusion, and functions in cell-to-cell spread of the virus (20, 21, 87, 113). Further, gB is an important immunogen eliciting both antibodies and cytotoxic T-cells in the host (72, 73).

The gB protein has been shown to contribute to both neurotropism and neurovirulence. Mutation of the HSV-1 gB resulted in altered clinicopathogenesis following inoculation of mice (48, 153). Replacement of gB in the neurovirulent HSV strain ANGpath with the gB coding sequence from the non-neurovirulent KOS strain substantially reduced the pathogenicity of ANGpath after peripheral inoculation of mice (83). Similarly, exchanging gB from the non-neurovirulent BHV-1 with gB from the neurovirulent PRV produced a BHV-1 mutant with increased neurovirulence and an altered route of neuroinvasion in young pigs (64).

The HVP2ap UL27 ORF consisted of 2685 nucleotides while the HVP2nv UL27 was slightly smaller at 2676 and 2682 nucleotides. The nucleotide in/dels that resulted in coding sequence size differences along with 4/9 subtype-specific amino acid changes were located in the putative N-terminal signal sequence. While the C-terminal cytoplasmic domain of gB is known to diverge between the simian viruses and HSV-1 (42) as well as between PRV and BHV viruses (87), no subtype-specific amino acid changes were located in this region of the HVP2 gB protein. The extremely low amino acid variance (0.04%) between HVP2 subtypes was somewhat surprising. Greater variance was expected between subtypes due to the large size of the gene and the fact that HSV-1 strains show an average of 0.08% variance between individual isolates. Due to the high degree of identity in amino acid sequence between the gB proteins from HVP2 subtypes, UL27 is an unlikely candidate to underlie the pathogenic differences between HVP2 subtypes in mice.

UL41 (Virion Host Shutoff)

The virion host shutoff (*vhs*) protein is the product of the UL41 gene. Homologues of the HSV-1 UL41 exist in most alpha-herpesviruses. The *vhs* is a constituent of the virion tegument and is expressed late in infection for packaging into nascent progeny virions.

Immediately following viral entry into the host cell, *vhs* is released into the cytoplasm whereby it rapidly and non-specifically degrades cellular mRNA prior to expression of viral proteins (51). Following initiation of viral transcription and translation, *vhs* is responsible for degrading viral mRNA as a means of regulating the switch between different kinetic classes of viral gene expression (114). Further, *vhs* plays a role in evading the anti-viral effects of type I interferons and other host cytokines via its ability to degrade host cell mRNA very early after infection (134). Finally, the HSV-1 *vhs* combines with ICP47 to block antigen presentation by MHC I and decrease expression of MHC II on infected cell surfaces (143). HSV *vhs* mutants are severely impaired in their ability to cause CNS disease in experimental animal model systems (7, 131-133).

A homologue of the HSV UL41 was recently identified in HVP2 strain X313 (13). Disruption of the X313 UL41 ORF diminished *vhs* RNase activity and the ability to shut off host cell protein synthesis in both Vero and primary baboon fibroblast cell cultures. In the current study, comparative sequencing of the HVP2 UL41 gene from both viral subtypes identified no subtype-specific amino acid differences.

To confirm that there was no difference in the expression of *vhs* between HVP2 subtypes, quantitative real-time PCR was used to determine the number of UL41 mRNA copies expressed at various time points following infection of PMDF cell cultures with 4.0 or 0.4 PFU/cell HVP2nv (OU1-76 strain) or HVP2ap (OU2-5 strain). As shown in Figure 3.4, there were no differences in numbers of UL41 transcripts present at any time point sampled following either high or low MOI infection. These results suggest that both HVP2 subtypes produce comparable numbers of UL41 transcripts so that it is unlikely to be a difference in *vhs* expression which determines the outcome of HVP2 infection in mice.

To verify that both HVP2 subtypes had RNase activity, northern blot analysis of infected PMDF and Vero cell cultures was performed. As shown in Figure 3.5, both HVP2nv- and HVP2ap-infected PMDF cell cultures and Vero cells had lower levels of β-actin mRNA detected at 4 hours PI relative to uninfected cells. This confirms that both HVP2 subtypes exhibit RNase activity and that there appears to be no difference in the *vhs* RNase activity based on cell type (i.e. Vero vs. PMDF). Further, there was no difference in the amount of cellular β-actin mRNA detected at 4 hours PI in HVP2nv- vs. HVP2ap-infected PMDF or Vero cells. Thus, *vhs*-mediated mRNA degradation does not seem to play a role in the differential replication of HVP2 subtypes *in vitro*. In conclusion, while it is possible that a function of *vhs* distinct from its RNase activity contributes to the disparate pathogenicity of HVP2nv vs. HVP2ap in mice, the similar kinetics of UL41 transcription coupled with the lack of apparent differential RNase activity for HVP2nv vs. HVP2ap implies that the HVP2 UL41 is not involved in the inefficient replication of HVP2ap subtypes observed in mice.

UL44 (Glycoprotein C)

The UL44 gene codes for glycoprotein C (gC). Although the exact functions of gC vary from virus to virus, gC or a homologous protein are produced by all alpha-herpesviruses. As a glycoprotein, gC is inserted into cell-derived membranes and is expressed as a late protein within infected cells. The gC protein is a major virus attachment protein and mediates primary attachment of the virion to the host cell by binding to heparan sulfate on the cell surface (23, 63, 94, 127). Additionally, in numerous alpha-herpesviruses gC has been shown to bind C' component C3 and its activation products (C3b, iC3b, and C3c) to block the binding of C5 and properdin to C3b, and to accelerate decay of the alternative C' pathway C3 convertase (10, 33, 47, 56, 57, 84). These mechanisms for gC-mediated C' interference represents yet another way in which the virus counteracts the early host immune response to infection.

HSV-1 gC-null viruses are significantly less pathogenic compared to wild-type HSV in mice and guinea pigs (89). In BHV-5 gC has been shown to be important for neurotropism and neurovirulence in rabbits (33).

The HVP2 gC protein sequence had more subtype-specific differences than any of the other HVP2 proteins examined with a total of 22 amino acid changes between the two virus subtypes. However, 1/9 conservative and 2/13 non-conservative amino acid differences were located in the signal sequence of the peptide and so are likely to have no effect on the mature, functional protein (Figure 3.6). Interestingly, 9/13 non-conservative amino acid differences were located in the membrane proximal region of the gC extracellular domain. Additionally, HVP2nv isolate OU1-76 had an insertion of 12

amino acids in this region. In HSV this domain is responsible for preventing binding of C5 and properdin to C3b (57, 84). The number and location of all cysteine residues were fully conserved between subtypes. With the exception of a single site in HVP2ap isolate OU2-5, all putative N-linked glycosylation sites (N-X-T/S) were fully conserved. This suggests that a strong structural similarity exists between the gC proteins from the two HVP2 subtypes despite the many amino acid differences.

A C' neutralization assay was performed to determine if the gC coding differences resulted in a differential sensitivity of HVP2nv vs. HVP2ap to C'-mediated neutralization. There was no difference in plaque size, morphology, or quantity of plaques produced between any of the HVP2 isolates treated with normal (C' intact) or heat inactivated (C' inactivated) baboon serum. These results led to the conclusion that, similar to HSV-1 (90), antibody-independent C' neutralization of the virus does not appear to inhibit the replication of either HVP2 subtype *in vivo*. Further, the additional amino acids in HVP2nv isolate OU1-76 do not appear to alter the ability of the virus to evade C'. These results together with the paucity of coding differences in other regions of the protein suggest that the HVP2 gC most likely does not contribute to the differential pathogenicity of HVP2 subtypes in mice.

Conclusions

Selected genes from HVP2nv and HVP2ap were sequenced in an attempt to identify coding differences in the HVP2 genome that could potentially account for the dichotomous pathogenicity of the two viral subtypes in mice (119). Candidate genes were chosen based on evidence for their involvement in neuroinvasiveness and/or

neurovirulence in other closely related alpha-herpesviruses. The genes examined are responsible for a wide range of viral functions: attachment to the host cell (gC and gB), cell-to-cell spread (US9, gE, and gI), viral replication (TK), regulation of viral gene expression (US11, ICP0, *vhs*), and evasion of the host immune response to viral infection (gE/gI complex, *vhs*, ICP0, gC, US12).

Comparative DNA sequencing revealed only very minor differences between HVP2nv and HVP2ap strains in the genes examined. As expected, the glycoproteins exhibited the highest variance at the amino acid level. These proteins are exposed on the exterior of the virus and as such are vulnerable to the host immune response. From a survival standpoint, the virus must be able to tolerate a certain degree of antigenic drift while maintaining the integrity and functionality of these proteins.

One interesting pattern that emerged from the sequence of several genes was that isolates from individual baboon facilities were more closely related to one another than to isolates of the same pathogenic phenotype. Also interesting was the sporadic nature of this phenomenon (ie. random genes throughout the genome rather than a block of sequence). This 'facility-specificity' was most evident in the US11 coding sequence where variable numbers of R-X-P reiterations correlated with distinct captive baboon colonies. Further, the RL2 ORF from HVP2nv isolate X313 and HVP2nv isolate A951 were more similar to one another than either isolate was to other isolates of their respective mouse-defined subtype. These isolates originated from the Southwest National Primate Research Center in San Antonio, TX although they are not believed to have been isolated at the same time. This colony is an older, well-established baboon

population that has not brought in animals from outside the colony in some time. In contrast, the OUHSC baboon colony was established much more recently and as such contains baboons accumulated from numerous facilities throughout the United States. This might explain why these facility-specific differences are more pronounced in isolates from colonies which have not imported animals into the colony for some time.

While the comparative sequencing approach failed to identify any major, readily identifiable sequence differences between HVP2 subtypes, minor changes in several genes may prove to be important as more information about the biology of HVP2 subtypes in cross-species infections is elucidated.

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Table 3.1

Origins of HVP2 Isolates

HVP2 Isolate	Colony of Origin	Baboon Species	Date of Isolation
A189164	WNPRC[1]	Unknown	Unknown
A951	SNPRC[2]	Unknown	1978
OU1-76	OUHSC[3]	Olive	Nov 1994
OU2-5	OUHSC	Olive	Dec 1995
OU2-9	OUHSC	Olive	Dec 1995
OU2-12	OUHSC	Olive	Dec 1995
OU2-13	OUHSC	Olive	Dec 1995
OU3-1	YNPRC/OU[4]	Yellow	June 1996
OU3-18	YNPRC/OU	Yellow	June 1996
OU3-40	YNPRC/OU	Yellow	June 1996
OU4-2	OUHSC	Chacma	Oct 1996
OU4-5	OUHSC	Olive	Oct 1996
OU4-8	OUHSC	Chacma	Oct 1996
OU5-35	OUHSC	Olive	May 2002
OU5-47	OUHSC	Yellow	May 2002
OU5-51	OUHSC	Yellow	May 2002
X313	SNPRC	Olive	1985-86
960	SNPRC	Olive	1985
1258	SNPRC	Olive	1985
1401	SNPRC	Olive	1985

Table adapted from reference 118

[1] Washington National Primate Research Center

[2] Southwest National Primate Research Center

[3] Oklahoma University Health Sciences Center

[4] Yerkes National Primate Research Center; baboons had been recently acquired from YNPRC by OUHSC; isolates were obtained while animals were in quarantine

Table 3.2

Comparative Sequence of Selected Genes from HVP2 Subtypes

Gene (protein)	Virus[1]	Size[2]		Subtype-specific NT differences[3]			
		NT	AA	S	CN	NC	Total
US7	HVP2ap/nv	1158	385	10	8	4	22
(gI)	HVP2nv (X313)	1161	386				
US8	HVP2nv	1620	539	17	6	13	36
(gE)	HVP2ap	1617	538				
	HVP2ap (OU2-5)	1611	536				
US9	HVP2nv/ap	276	91	0	0	0	0
US11	HVP2nv	432	144	0	0	0	0
	HVP2nv (X313)	531	177				
	HVP2ap	441	147				
	HVP2ap (A951)	531	177				
US12	HVP2nv/ap	237	78	0	0	0	0
UL23 (TK)	HVP2nv/ap	370	1113	4	2	3	9
UL27	HVP2nv	2676	891	5	3	6	14
(gB)	HVP2nv (X313)	2682	893				
	HVP2ap	2685	894				
UL41 (*vhs*)	HVP2nv/ap	1446	481	6	0	0	6
UL44	HVP2nv	1389	462	30	9	13	52
(gC)	HVP2nv (OU1-76)	1428	476				
	HVP2ap	1398	466				
RL2	HVP2nv/ap	1722	573	1	0	0	1
(ICP0)[4]	HVP2nv (OU1-76)	1680	559				
	HVP2nv (A189164)	1668	555				
	HVP2ap (OU2-5)	1713	570				
	HVP2ap (OU4-8)	1701	566				

[1]Individual viral isolates are grouped by HVP2-subtype (nv or ap); where an individual HVP2 isolate varied in size, the variant virus is listed

[2] Number of nucleotides (NT) and amino acids (AA) for HVP2 CDS

[3] Types of amino acid differences: silent (S), conserved amino acid change (CN), and non-conserved (NC) amino acid change

[4] Number of nucleotides in RL2 CDS

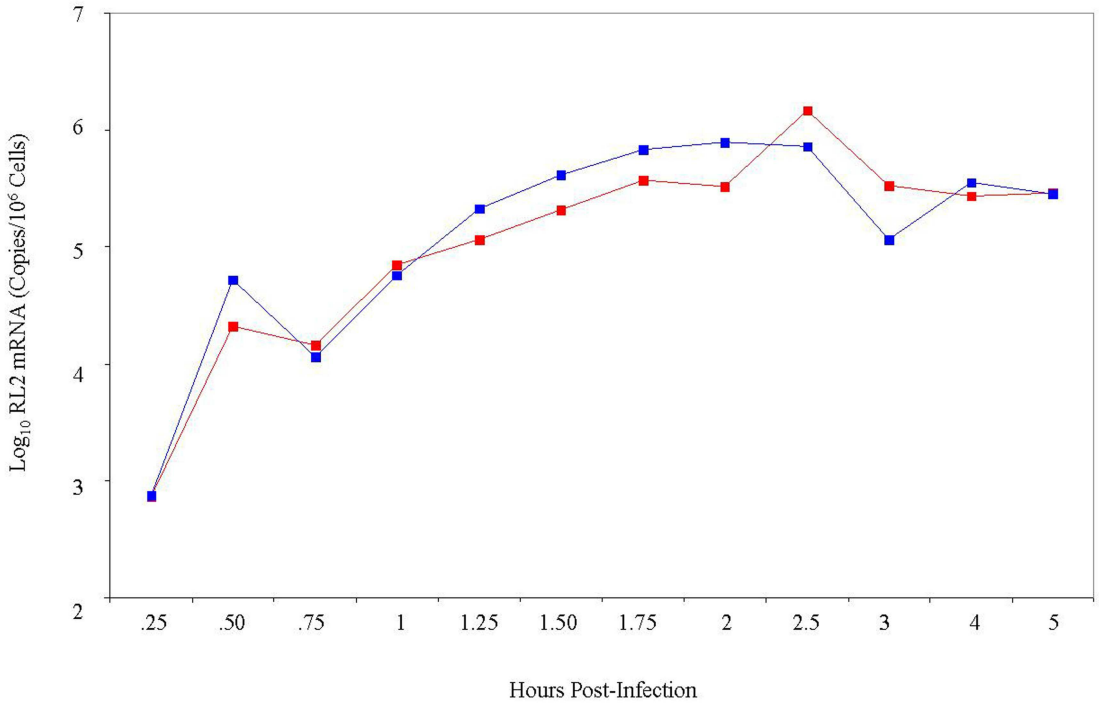

Figure 3.1 – Real time PCR quantitation of HVP2 RL2 mRNA in HVP2-infected PMDF cell cultures. Confluent PMDF cell cultures in 12-well trays were infected with 4.0 PFU/cell of HVP2nv (■) or HVP2ap (■). At designated time points PI, cells were harvested and total RNA extracted and purified. Following reverse transcription, 5.0 µl of cDNA was used in a quantitative real-time PCR assay using HVP2-specific primers and a Taqman® MGB probe to determine the number of RL2 transcripts present in the original sample. Data points represent mean copy numbers of RL2 mRNA/10^6 cells determined in triplicate real-time PCR reactions.

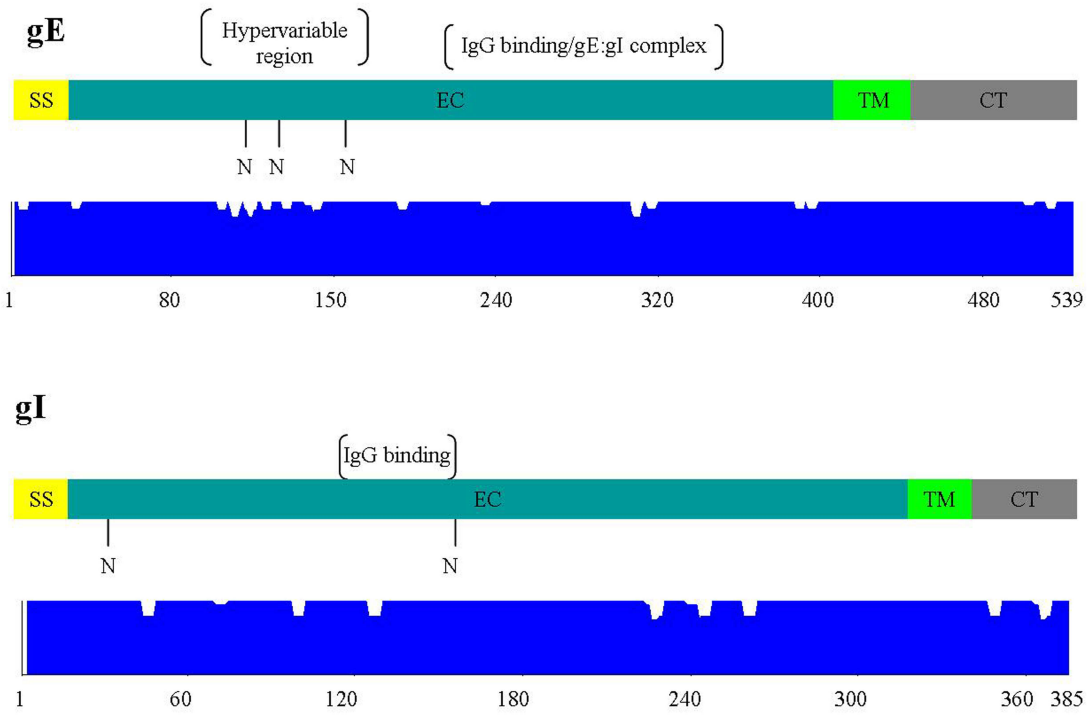

Figure 3.2 - HVP2 gE and gI protein domains and similarity profiles. ORFs for both gE and gI were identified in both HVP2 subtypes. The similarity profile diagrams (bottom) show the location of subtype-specific amino acid changes observed between HVP2ap and HVP2nv in relation to protein domains in which the differences were located. The graphical representation (top) details approximate domains for the signal sequence (SS), extracellular domain (EC), transmembrane domain (TM), and cytoplasmic tail (CT) as well at putative N-linked glycosylation sites (N). The protein regions implicated in HSV-1 gE:gI complex formation and monomeric IgG binding and the hypervariable region identified in HSV gE are also indicated.

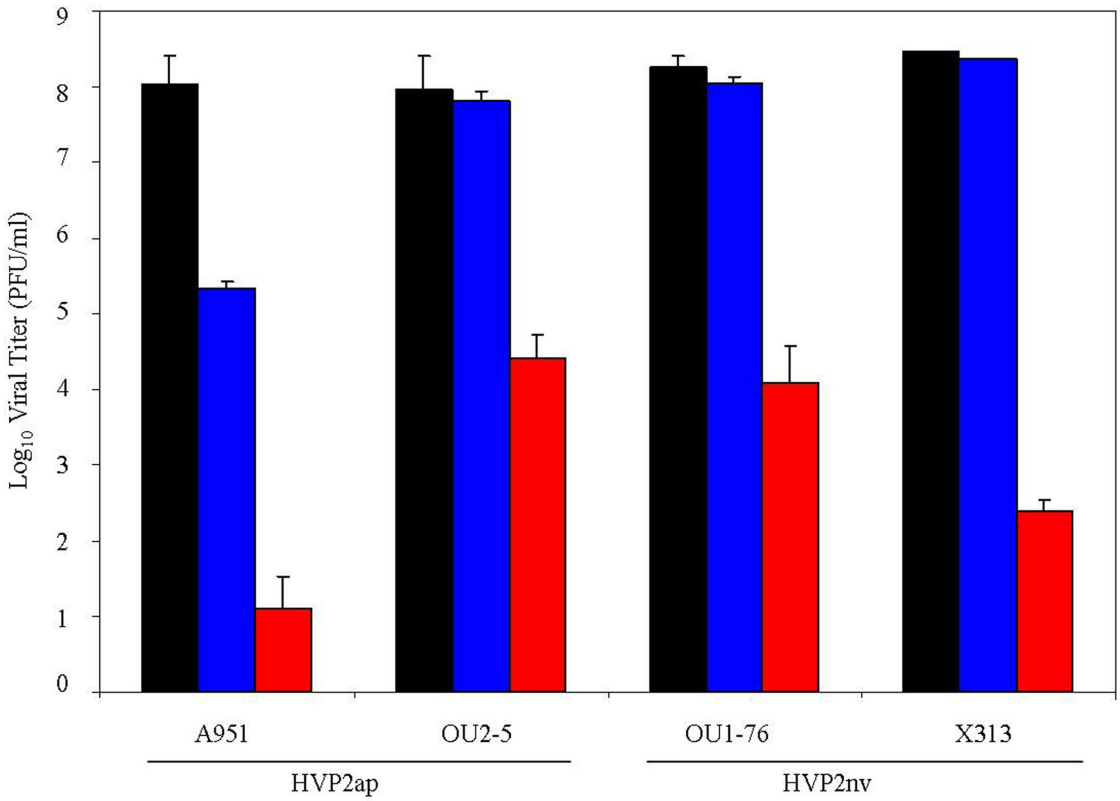

Figure 3.3 - ACV sensitivity of HVP2 isolates. Vero cell monolayers were infected with 1.0 PFU/cell of HVP2nv (OU1-76 and X313) or HVP2ap (OU2-5 and A951). After one hour cells received fresh media (■) or fresh media containing 2.0 (■) or 20.0 (■) µg/ml of ACV and incubated at 37 °C for a total of 24 hours. Data points represent mean PFU/ml values of replicate experiments (n=4). While all HVP2 isolates tested were sensitive to 20 µg/ml of ACV, only HVP2ap isolate A951 was sensitive to treatment with 2.0 µg/ml. Treatment of the cells with DMSO (solvent) did not inhibit replication of HVP2 (data not shown).

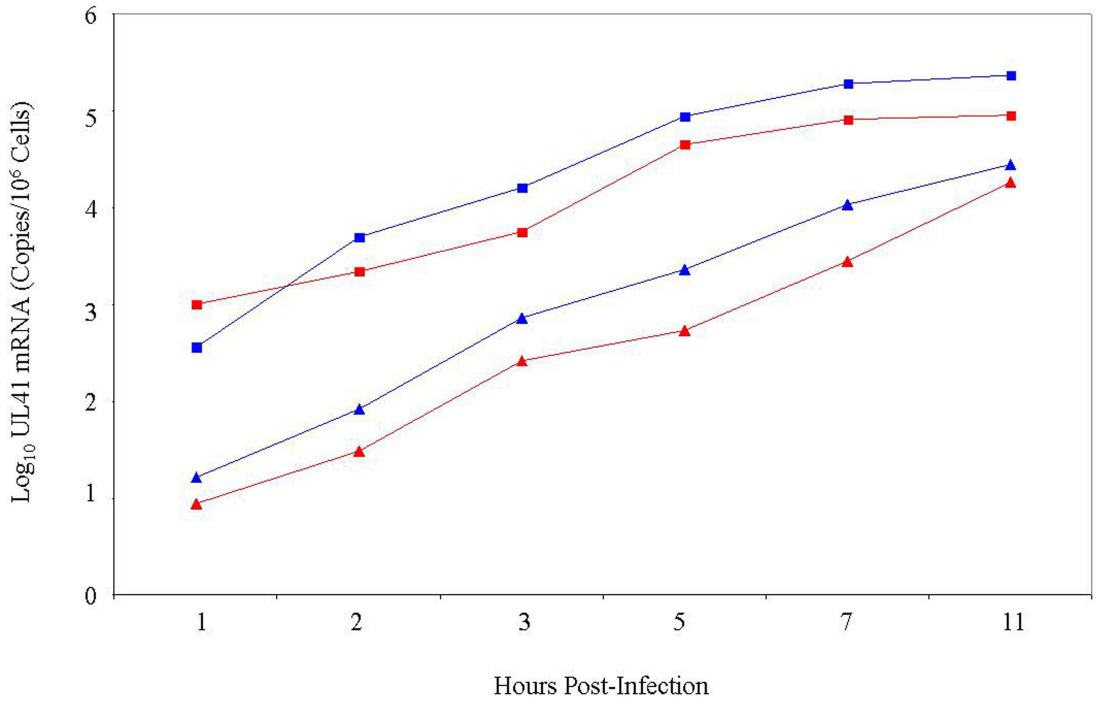

Figure 3.4 - Real time PCR quantitation of HVP2 UL41 mRNA in HVP2-infected PMDF cell cultures. Confluent PMDF cell cultures in 12-well trays were infected with 0.4 (▲) or 4.0 (■) PFU/cell of HVP2nv or HVP2ap. At designated time points PI, cells were harvested and total RNA extracted and purified. Following reverse transcription of total RNA, 5.0 µl of cDNA was used in a quantitative real-time PCR assay with HVP2-specific primers and a Taqman® MGB probe to determine the number of UL41 transcripts in the original sample. Data points represent mean copy numbers of UL41 mRNA/10^6 cells determined in triplicate real-time PCR reactions.

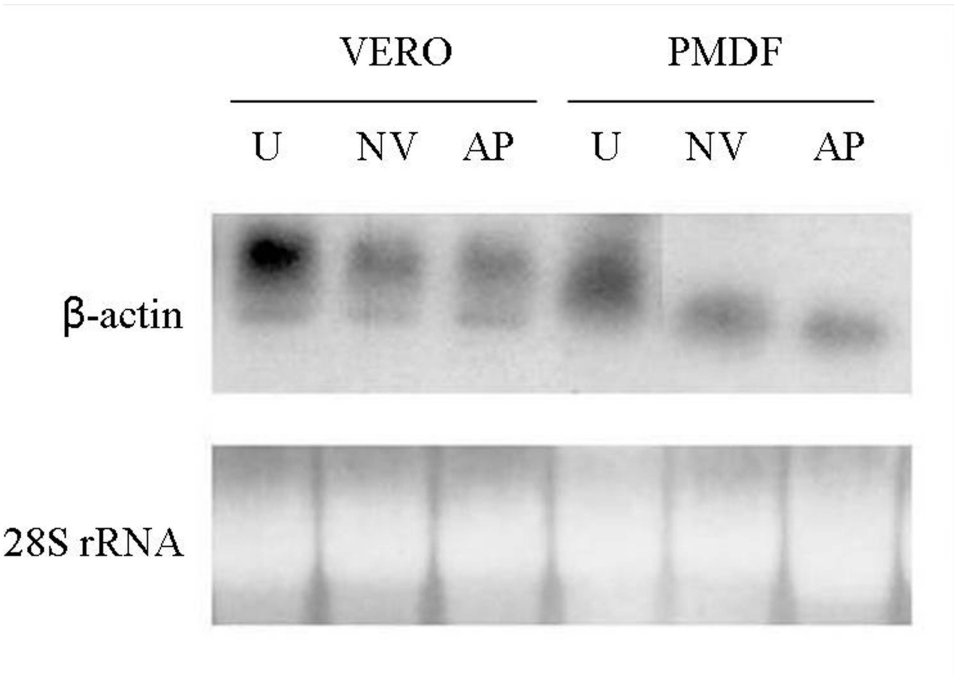

Figure 3.5 - Comparative RNase activity of HVP2 *in vitro*. At 4 hours PI, total RNA was isolated from Vero cell cultures or PMDF cell cultures mock infected with sterile PBS (U) or infected at an MOI of 1.0 PFU/cell with HVP2nv isolate OU1-76 (NV) or HVP2ap isolate OU2-5 (AP). Equivalent samples consisting of 15 ng of total RNA were analyzed by northern blot analysis using a ^{32}P-labeled β-actin probe (top). Relative amounts of β-actin mRNA were determined by autoradiography. Comparable levels of 28S rRNA in each sample were confirmed by staining the agarose gel with ethidium bromide (bottom). There was no difference in the amount of β-actin mRNA present in HVP2nv- vs. HVP2ap-infected Vero or PMDF cell cultures.

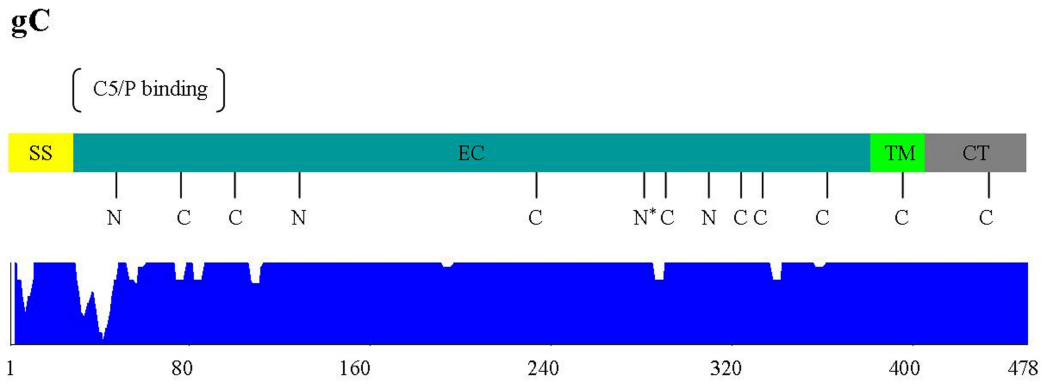

Figure 3.6 - HVP2 gC protein and subtype similarity profiles. The similarity profile (bottom) diagrams the subtype-specific amino acid changes observed between HVP2ap and HVP2nv. The graphical representation of the gC protein (top) aligns with the similarity profile and identifies locations of the signal sequence (SS), extracellular domain (EC), transmembrane domain (TM), cytoplasmic tail (CT), conserved cysteine residues (C), and putative N-linked glycosylation sites (N). One proposed N-linked glycosylation site was not conserved in HVP2ap OU2-5 strain (N^{*}). The protein region shown to inhibit the binding of C' components C5 and properdin (P) to C3b in HSV-1 gC are also indicated.

CHAPTER IV

EXPERIMENTAL INFECTION OF BABOONS (*Papio cynocephalus anubis)* WITH APATHOGENIC AND NEUROVIRULENT SUBTYPES OF *Herpesvirus papio* 2

Summary

Cercopithecine herpesvirus 16 [Herpesvirus papio 2; HVP2] is an α-herpesvirus of baboons (*Papio spp*.) which generally causes minimal to inapparent disease in the natural host species. HVP2 is very closely related genetically and antigenically to *Cercopithecine herpesvirus* 1 [monkey B virus; BV] of macaques which is well known for its extreme lethality in non-macaque species including humans. Preliminary evidence suggests that an HVP2/mouse model would be an excellent tool for studying zoonotic BV infections. While the pathogenicity different BV isolates in mice covers the full range of severity from apathogenic to extremely neurovirulent, testing of multiple HVP2 isolates revealed only two distinct phenotypes in mice regardless of route of inoculation: apathogenic (HVP2ap) or highly neurovirulent (HVP2nv). For the HVP2nv/mouse model to truly reflect BV infection in both its natural host and the differential pathogenicity of BV in aberrant host species, HVP2nv should not produce severe disease in its natural host. To test this, juvenile baboons were inoculated with doses of 10^6 or 10^4 PFU of HVP2ap or HVP2nv using an oral subdermal inoculation route. Parameters of interest included the appearance of lesions, shedding of infectious virus, general health,

and the host humoral response to the infection. Regardless of the inoculum dose used, there were no differences noted between the two HVP2 subtypes in baboons in any of the parameters measured. These findings further support the use of the HVP2nv/mouse system as a model to elucidate and study the viral determinants associated with cross-species BV neurovirulence.

Introduction

Cercopithecine herpesvirus 16 [Herpesvirus papio 2; HVP2] is a ubiquitous pathogen of both wild-caught and captive-born baboons (*Papio spp*.) that is very closely related to herpes simplex virus types 1 and 2 (HSV1 and HSV2) of humans (4, 5). Like most of the simian α-herpesviruses, much of what is assumed about the biology of HVP2 infections in baboons is actually extrapolated from what is known of HSV in humans. Recently, Payton et al (2004) showed that HVP2 is acquired by captive infant baboons as an oral infection by infants as juveniles or as a genital infection in sexually mature animals. A hallmark of α-herpesviruses in their natural host is the ability to establish a latent infection in sensory ganglia with periodic reactivation and shedding of infectious virus. Lesions, resulting from primary or recurrent infection, may not be readily evident. Like HSV2 in humans, neonatal HVP2 infection can produce lethal generalized infections (1, 6, 7, 22). Thus, in many respects the biology of HVP2 in baboons is similar to HSV in humans.

HVP2 is also very closely related to *Cercopithecine herpesvirus* 1 [monkey B virus; BV] of macaques (*Macaca sp*.). BV is well known for its extreme neuroinvasiveness and lethality when transmitted to humans and other non-macaque

primate species. Despite the high degree of genetic and antigenic relatedness of BV and HVP2, there have been no reported cases of human HVP2 infections (16, 20). How these two viruses can be so similar genetically and yet apparently exhibit such dichotomous zoonotic potential is a question that needs to be addressed.

Several studies have examined the mouse as a potential small animal model system with which to study the neurovirulence of BV (16, 20). In mice, the pathogenicity of different strains of BV varies widely across a full spectrum of virulence phenotypes from completely apathogenic to extremely neurovirulent (17). In contrast, experiments in mice testing multiple HVP2 isolates revealed quite a different picture in that all isolates tested fall into one of two distinct subtypes: apathogenic (HVP2ap) or highly neurovirulent (HVP2nv) (20). All HVP2nv isolates tested to date produce a fulminant, fatal, CNS infection and invoke a robust anti-HVP2 serum IgG response while HVP2ap causes no clinical signs of disease and evokes a minimal IgG response compared with HVP2nv (20). Furthermore, the neuroinvasiveness and neurovirulence phenotypes of HVP2nv remain consistent when the virus is administered to mice using different modes of inoculation including intra-cranial, intra-muscular, and skin scarification (18-20). In addition, HVP2nv infections in mice resemble those induced by the most pathogenic strains of BV in severity and fatality. In addition to those listed above, there are several other factors which make the HVP2nv/mouse system an excellent model system for zoonotic BV infections: the existence of the non-neurovirulent HVP2ap subtype and the fact while BV is the only biosafety level (BSL) 4 herpesvirus, HVP2 only requires BSL-2 precautions making it significantly safer and easier to work with experimentally

compared to BV. Thus, the HVP2nv/mouse system appears to be an excellent, consistent, and reproducible model for studying zoonotic BV infections.

There is no evidence to suggest that severity of HSV infection in humans corresponds to a particular viral strain or isolate. For example, no single HSV strain is more or less associated with human neonatal CNS infections than any other HSV strain. In contrast, isolates of HSV behave very differently from one another in various experimental non-natural hosts. In further examining the usefulness of the HVP2nv/mouse model for zoonotic BV infections, one very pertinent question arose - Do the two HVP2 subtypes exhibit differences in the natural host species? Differences between the HVP2 subtypes in baboons would negate the use of the HVP2/mouse model as it would suggest that differences exist between HVP2ap and HVP2nv which are not specific to cross-species infections. Therefore, it was the goal of the current study to compare HVP2nv to HVP2ap within its natural host species.

Materials and Methods

Animals

Juvenile olive baboons (*Papio cynocephalus anubis*) from the specific pathogen free (SPF) program at University of Oklahoma Health Sciences Center (OUHSC) were used in this study (Table 4.1). Within 12 hours of birth, infants were removed from their dams, disinfected, and subsequently hand-reared in the AAALAC-accredited OUHSC SPF barrier facility. Periodically infants were tested serologically by ELISA for eleven viruses: HVP2, *C. herpesvirus* 15 (*Herpesvirus papio* 1; HVP1), *C. herpesvirus* 8

(baboon cytomegalovirus;CMV), *C. herpesvirus* 17 (baboon rhadinovirus), *C. herpesvirus* 9 (simian varicella virus), simian T lymphotrophic virus, simian foamy virus, simian retrovirus, simian immunodeficiency virus, simian virus 40, and measles. Animals with a positive titer to any target viruses were immediately culled from the SPF group and moved out of the SPF facility where they remained isolated from conventional animals. All baboons used in this study were culled from the SPF colony because they had seroconverted to either CMV or HVP1; however, prior to these experiments, all culled baboons had been housed together so that most animals had seroconverted to both viruses (Table 4.1).

Viruses and Cells

Vero cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM containing 2% FBS. The origins of HVP2nv isolates OU1-76 and X313 and HVP2ap isolates OU2-5 and A951 have been previously described (3-5, 20).

Baboon Inoculations

All protocols were approved by the OUHSC Institutional Animal Care and Use Committee. All baboons were sedated with 5 - 10 mg/kg Ketamine HCl (Ketaved™, Vedco Inc., St. Joseph, MO, USA) given intra-muscularly. Baboons in groups of two or three were inoculated intradermally in oral mucosa of the bottom lip, near the junction with the exterior keratinized epithelium with 50 µl of HVP2ap or HVP2nv. Doses of 10^4 plaque forming units (PFU) or 10^6 PFU were injected using a 1 cc syringe and a 27 ½-

gauge needle. Sterile PBS was used as diluent for all viral dilutions and as inoculum for a group of two negative control animals.

Sample Collection

Prior to inoculation and at designated time-points out to 21 days post-infection (DPI) animals were sedated for weighing, swabbing, obtaining a rectal temperature, and drawing blood. Blood was drawn from a femoral artery for serology and complete blood count (CBC) using a six ml syringe with a 21-gauge needle. Serum samples were stored at -80°C until tested by ELISA to detect anti-HVP2 antibody. For CBCs approximately 1-2 ml of blood was collected into EDTA and placed on a blood mixer for five minutes before evaluation with a VetABC – Diff hematology analyzer (Heska, Fort Collins, CO, USA).

All animals had both the site of inoculation and oral pharynx swabbed with sterile cotton-tipped swabs which were then immediately placed into DMEM prepared as described previously (5) and stored at -80° C. Swab samples were processed and inoculated onto Vero cells for virus isolation as previously described (5).

Immunoassays

ELISA procedures for detection of serum anti-HVP2 antibodies were basically as described (15) except that peroxidase-conjugated anti-human IgG or IgM (Vector Laboratories, Burlingame, CA) were used as secondary antibodies at dilutions of 1:5000 and 1:250, respectively. All baboon sera were tested at a 1:100 dilution.

Polymerase Chain Reaction (PCR)

Template DNA for PCR was obtained by harvesting Vero cells which had been inoculated with swab samples. The cells were scraped into the media and harvested when the CPE was complete. Total DNA was extracted using the DNAeasy Tissue Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's protocol, quantitated by spectroscopy, and stored at -20° C.

PCR reaction mixtures contained 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 5% DMSO, 10% betaine (Sigma Aldrich Co., St. Louis, MO, USA), 250 U *Taq* polymerase, and 25 pM each primer. Primer sets for the US8 (glycoprotein E) gene (603F 5'-CTACGTCATGGAGGTGGTCTGGG-3'; 604R 5'-GACGCCGAGGGAGACGTCGGGCGC-3') and the UL23 (thymidine kinase) gene (614F 5'-CATCTCCCGGGGCGAATTCGCG-3'; 615R 5'-GGTCGCCAAGCACGTGCGCCTG-3') were purchased from Sigma Genosys (The Woodlands, TX, USA). All PCR reagents were obtained from Applied Biosystems, Foster City, CA, USA unless otherwise specified. After a five minute denaturation at 94°C, amplification was carried out by 35 cycles of [94°C-30 sec/58°C-30 sec/72°C-1.5 min] followed by seven minutes at 72°C. PCR products were purified prior to sequencing using Wizard PCR Preps (Promega, Madison, WI, USA) and sequenced with the same primers used for the PCR reaction. All sequencing was done at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA). Vector NTI Suite 8.0 (InforMax, Inc., Frederick, MD, USA) was used to assemble and analyze sequence files.

Results

To determine whether the HVP2 subtypes which have been defined in a mouse model exhibit any differences in the natural host species, juvenile baboons with an average age of 1.8 years were infected by intradermal inoculation of the oral mucosal epithelium. This method of inoculation was chosen over topical application of the virus to allow consistent, reproducible administration of a defined dose of the virus

Clinical Signs of HVP2 Infection

The nature of the lesions was similar to those observed in other oral α-herpesvirus infections (Figure 4.1) (10, 21). Several baboons developed lesions at secondary sites such as the tip of the tongue, the upper lip, or the gums. A single animal in the OU2-5 10^6 PFU dosage group developed a lesion on the external skin adjacent to the internal site of inoculation. No lesions were observed in the oropharyngeal region in any of the animals.

Following inoculation with 10^6 PFU of either HVP2nv (OU1-76) or HVP2ap (OU2-5), primary lesions averaging 1-2 mm were observed at the site of inoculation by 2 DPI. Lesions increased in severity until 7 DPI, some reaching 5-7 mm in diameter before starting to heal (Figure 4.1). In contrast, only 2/3 and 1/3 animals inoculated with 10^4 PFU of HVP2nv isolate OU1-76 or 10^4 PFU of HVP2ap isolate OU2-5, respectively, developed lesions at the site of inoculation. These lesions did not appear until 4-5 DPI and were much smaller in size, averaging 0.5-1mm in diameter at their most severe. Animals inoculated with 10^4 PFU of HVP2ap (A951) or HVP2nv (X313) developed

lesions starting at approximately 3-4 DPI with an initial size range of 2-3 mm in diameter. These lesions continued to increase in diameter, some reaching 5-7 mm before beginning to subside. One baboon inoculated with 10^4 PFU of X313 did not develop any lesions over the course of the experiment. In all animals the lesions had completely healed by the end of the experiment.

Throughout the experiment, all animals continued to eat normally and no weight loss was measured; instead, these baboons continued to grow normally (data not shown). For all animals rectal temperatures remained within the normal range throughout the experiment. All CBC values fell within the normal range throughout the course of the experiment and no differences were observed between values for animals inoculated with HVP2nv or HVP2ap.

Virus Isolation and DNA Sequencing

Once inoculated, both the site of inoculation and the oropharynx were swabbed at regular intervals to determine how long after inoculation each animal shed infectious virus. The results of virus isolation are detailed in Table 4.2. At a dose of 10^6 PFU, both HVP2 subtypes were readily isolated from the site of inoculation and the oropharyngeal region over the 21 day experiment. However, at the lower dose of 10^4 PFU of the same two isolates (OU2-5 and OU1-76), HVP2ap was isolated from only 2/3 animals and then only rarely (1/12 and 2/12 days) and only from the site of inoculation. Interestingly, the duration of viral shedding from animals inoculated with 10^4 PFU of HVP2nv isolate X313 and HVP2ap isolate A951 more closely resembled that seen in the baboons that received 100-fold higher doses of the OU1-76 and OU2-5 isolates. In all groups, virus

shedding did not correlate with the presence or absence of observable lesions. There was also no correlation between the HVP2 subtype and the pattern of viral shedding following inoculation.

To confirm that the virus isolated from each animal was the same as the HVP2 strain used as inoculum, DNA prepared from virus recovered from each animal was amplified by PCR and sequenced. For each animal, sequences amplified from the recovered virus were 100% identical to the HVP2 isolate the animal had been inoculated with.

Serology

Serum was collected over the course of the experiment to monitor anti-HVP2 IgG and IgM seroconversion following infection. Baseline serum samples were obtained prior to inoculation and all animals were confirmed to be seronegative for both anti-HVP2 IgM and IgG.

Regardless of the HVP2 subtype, baboons that received 10^6 PFU of HVP2 first developed detectable amounts of serum anti-HVP2 IgM by 4-7 DPI while anti-HVP2 IgM first appeared 7-11 DPI in animals that received 10^4 PFU. (Figure 4.2A). All animals that developed detectable anti-HVP2 IgM levels showed decreased titers at 21 DPI. Of the 14/16 baboons that seroconverted, all had detectable serum anti-HVP2 IgG titers by 7-11 DPI regardless of the viral dose given (Figure 4.2B). Two baboons did not have detectable anti-HVP2 IgM or IgG over the course of the experiment: one received 10^4 PFU of HVP2nv isolate X313 while the second was inoculated with 10^4 PFU of

HPV2ap isolate OU2-5. Overall, there were no temporal or quantitative seroconversion differences noted between HVP2ap- and HVP2nv-inoculated animals.

Discussion

The course of a typical α-herpesvirus infection in its natural host species has been very thoroughly studied and well documented for several viruses including HSV1 and HSV2 of humans (11), bovine herpesviruses types 1 and 5 in cattle (14), and porcine pseudorabies virus (13). Based on the high degree of genetic and antigenic similarity between human and simian herpesviruses, it has been assumed that within the context of the natural host species, both clinical manifestations of and the immune response to primary and recurrent simian α-herpesvirus infections are the same as in humans. Where specific parameters of HVP2 infection in baboons have been examined, the results have largely affirmed the validity of this assumption. Kalter et al (1978) isolated HVP2 from the trigeminal ganglia of apparently healthy baboons, thus demonstrating that HVP2 is able to establish and maintain latency within the sensory ganglia of its natural host species. Much like HSV, HVP2 has been isolated from animals that are shedding either asymptomatically or in the presence of oral and/or genital lesions (5, 12). Experimental infections using intravenous or intra-tracheal inoculation of HVP2 have demonstrated a severe systemic infection in newborn baboons similar to human neonatal HSV2 infections, while older slightly older baboons exhibited no clinical signs of infection (1, 6, 7). Recently, a naturally occurring HVP2nv infection was shown to be the etiological agent in a fatal case of neonatal pneumonia in a baboon infant destined for the OUHSC SPF program (22). The HVP2nv isolate OU1-76 was isolated from an oropharyngeal

swab taken from an infant recovering from a respiratory tract infection of undetermined etiology (5). Finally, similar to HSV, the major antigenic proteins of HVP2 (glycoprotein D and glycoprotein B) represent consistent targets for the host immune response (4). Thus, HVP2 infections in baboons are in many ways very similar to HSV infections in humans.

While we did observe differences in the severity of lesions produced by different isolates of HVP2, these did not correlate to their mouse phenotype. The HVP2nv strain X313 was isolated during an HVP2 outbreak in a captive baboon colony in 1985 (12) while the HVP2ap strain A951 was isolated from the trigeminal ganglion of an asymptomatic baboon in the late 1970's (9). In contrast to the X313 and A951 isolates which had been passaged multiple times in tissue culture, the OU1-76 and OU2-5 isolates were isolated more recently and low-passage preparations of these isolates were available for this study. Unexpectedly, it was the older, high-passage isolates that produced more severe lesions and more prolonged shedding of virus. The infection produced by these two isolates appeared similar to that observed with a 100-fold higher dose of the low-passage isolates. While *in vitro* passage appears to decrease the neurovirulence of SA8, a closely related vervet virus, in different lab animal species (2, 8), we are not aware of any studies that have directly assessed the virulence of high-passage isolates in the natural host species. In addition, HVP2nv isolate X313 has retained the extreme neurovirulence for mice through a high number of tissue culture passages (16, 20). Taken together, it seems that tissue culture adaptation of HVP2 does not correlate with either the zoonotic potential or the pathogenicity of the virus in its natural host.

Two animals in the study did not develop detectable anti-HVP2 IgG or IgM levels following inoculation. One animal in the HVP2nv (X313) 10^4 PFU dosage group developed an erythema at the site of inoculation 3-5 DPI which coincided with the isolation of virus from both the oral and oropharygeal swabs at 4-5 DPI. This indicates that the animal was infected but that infection was efficiently controlled by the innate immune system. It is interesting to speculate that the animal's age may have played a role in the immune response to the virus as this animal at 2.6 years of age was the oldest baboon used in this study (six months older than the next oldest baboon). In contrast, the second animal that did not seroconvert (inoculated with 10^4 PFU of HVP2ap strain OU2-5) did not develop any lesions but was positive for virus isolation from the site of inoculation at 18 DPI. The lack of both lesions at the site of inoculation and detectable levels of serum anti-HVP2 IgM or IgG coupled with the single, spurious isolation of virus suggests that experimental error may have resulted in inoculation with less than 10^4 PFU in this animal and that the single positive swab sample resulted from contamination during collection or processing can not be ruled out. Alternatively, it is possible that this particular animal was resistant to the HVP2 infection as the result of a stronger innate immune response.

While HVP2 strains can be readily divided into two distinct subtypes based on their pathogenicity in mice, no such pattern was observed in baboons. Although no differences were seen between HVP2ap and HVP2nv when 10^6 PFU was administered, such a high dose could have been sufficient to overwhelm the host defenses. However, 100-fold less virus also failed to reveal any difference between the two subtypes. Having obtained the same results with two additional, unrelated, HVP2 strains, we are confident

in concluding that the two mouse-defined subtypes of HVP2 do not exhibit significant pathogenic differences within their natural host. This provides further evidence that the HVP2/mouse system is a particularly good model system for elucidating the viral determinants that allow some herpesviruses to produce highly lethal CNS infections when transmitted to a non-natural host species despite producing a comparatively mild infection in their natural host species.

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Table 4.1

Baboon Data

Animal ID	Sex	Age (years)	Herpesvirus status[a]	
			CMV	HVP1
6802	F	1.8	+	+
7402	F	1.8	+	+
603	M	1.9	+	+
5803	F	1.3	+	-
7502	M	2.0	+	+
3303	M	1.8	+	+
4303	M	1.7	+	+
7302	M	2.0	+	+
403	F	1.7	+	-
703	F	1.7	+	-
203	M	1.9	+	+
6702	M	2.1	+	+
5203	F	1.4	-	+
6303	M	1.3	+	+
3703	F	1.7	+	+
4902	M	2.6	+	+

[a]All animals were seronegative for all other known baboon herpesviruses

Table 4.2

Virus Isolation from Oral Swabs

HVP2 Strain/ Dose (PFU)	Animal ID	Virus Isolation from Swabs (Site/Oropharynx)[a]											Total Positive Samples[b]	
		DPI												
		2	3	4	5	7	9	11	14	16	18	21	Site	Oropharynx
HVP2ap														
OU2-5 10^6	6802	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+/+	11	10
	7402	+/+	+/+	+/+	+/+	+/+	-/-	-/+	-/-	-/-	-/-	-/-	5	6
OU2-5 10^4	603	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	0
	5803	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	+/-	-/-	-/-	2	0
	7502	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	1	0
A951 10^4	3303	-/-	-/-	-/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	2	3
	4303	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-	6	7
	7302	-/-	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	5	4
HVP2nv														
OU1-76 10^6	403	+/+	+/+	+/+	+/+	+/+	-/+	+/-	-/-	-/-	-/-	+/-	8	7
	703	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/+	-/+	-/-	-/-	5	5
OU1-76 10^4	203	-/-	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	+/-	+/-	5	3
	6702	-/-	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	6	5
	5203	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	1	0
X313 10^4	6303	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	5	5
	3703	+/+	+/+	-/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	5	5
	4902	-/-	-/-	+/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	2

[a] Results of virus isolation from swabs taken from both the site of inoculation and oropharynx

[b] Number of swab samples which were positive for HVP2 isolation on Vero cells; a total of 11 swabs were obtained for each region swabbed from each animal.

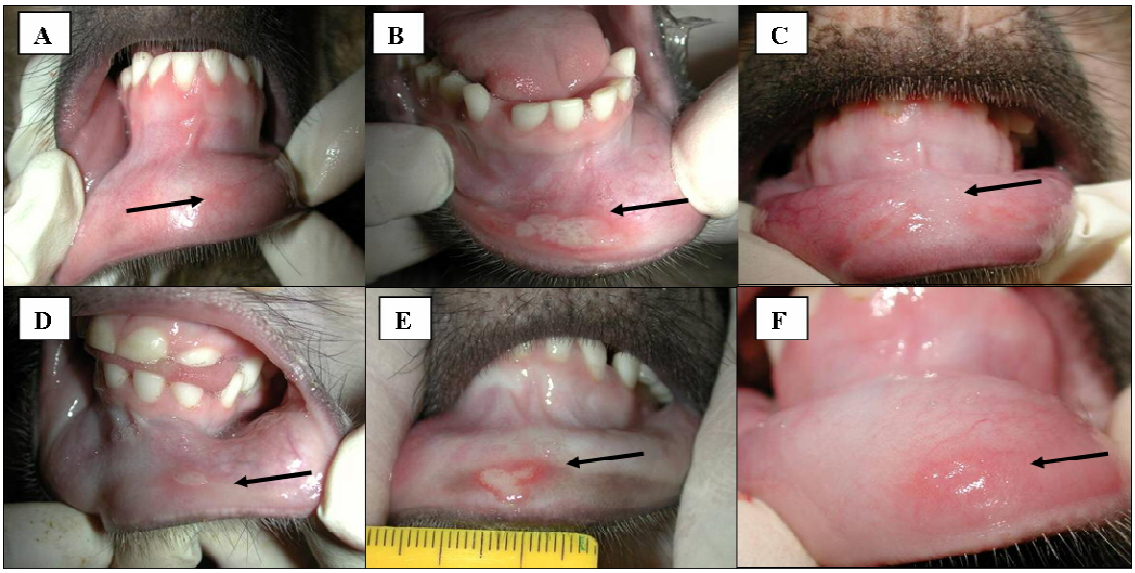

Figure 4.1 - Lesions at the site of inoculation following HVP2 infection. No differences were noted in the time of onset, severity, or duration of oral lesions in baboons infected with 10^4 PFU of HVP2ap (Panels A-C) or HVP2nv (Panels D-F). Following inoculation, erythema developed at the site of inoculation which progressed to vesicles or pustules by 3-4 DPI (Panels A and D). In some cases multiple vesicles formed which coalesced to form a large ulcer. By approximately 7 DPI the vesicles ruptured to form ulcerative lesions (Panels B and E) which rapidly healed by 14 DPI (Panels C and F) leaving no permanent scarring. No lesions were observed in control animals over the course of the experiment.

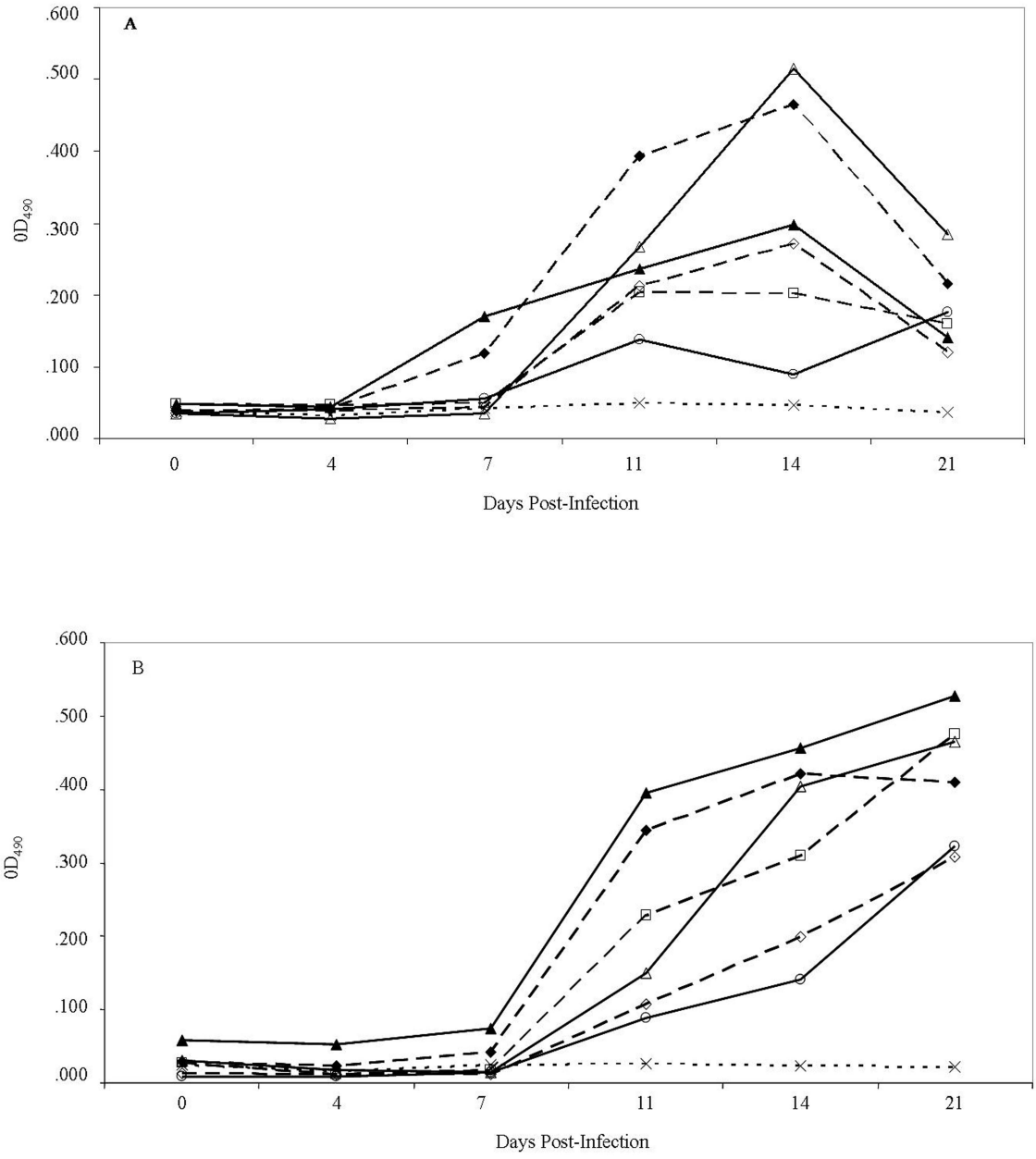

Figure 4.2 - Seroconversion of baboons during primary HVP2 infection. Anti-HVP2 serum IgM (A) and IgG (B) levels were measured by ELISA at 0, 4, 7, 11, 14 and 21 DPI. All sera were tested at a 1:100 dilution and peroxidase-conjugated anti-human IgG or IgM were used as secondary antibodies. Solid lines represent the average OD for animals inoculated with HVP2nv (OU1-76 10^6▲/10^4Δ; X313 10^4 ○) while dotted lines

represent the average OD for animals inoculated with HVP2ap (OU2-5 10^6 ♦/ 10^4 ◊; A951 10^4 □) or control animals (----x----).

CHAPTER V

NEUROPATHOGENESIS OF *Herpesvirus papio* 2 IN MICE PARALLELS *Cercopithecine herpesvirus* 1 (B VIRUS) INFECTIONS IN HUMANS

Summary

Cercopithecine herpesvirus 1 (monkey B virus; BV) produces extremely severe and usually fatal infections when transmitted from macaque monkeys to humans. *C. herpesvirus* 16 (*Herpesvirus papio* 2; HVP2) is very closely related to BV yet cases of human HVP2 infection are unknown. However, following intra-muscular inoculation of mice HVP2 rapidly invades the peripheral nervous system and ascends the central nervous system resulting in death very much like human BV infections. In this study we further evaluated the neurovirulence of HVP2 in mice as a potential model system for human BV infections. HVP2 was consistently neurovirulent when administered by epidermal scarification, intra-cranial inoculation and an eye splash. Quantitative real-time PCR, histopathology, and immunohistochemistry were used to follow the temporal spread of virus following skin scarification and to compare the pathogenesis of neurovirulent and apathogenic isolates of HVP2. Apathogenic isolates were found to be capable of reaching the CNS but were extremely inefficient at replicating within the CNS. We conclude that neurovirulent strains of HVP2 exhibit a pathogenesis in mice that parallels that observed in human BV infections, and that this model system may prove

useful in dissecting the viral determinants underlying the extreme severity of zoonotic BV infections.

Introduction

Cercopithecine herpesvirus 1 (monkey B virus; BV) is a ubiquitous pathogen of macaques (*Macaca spp*.). In captive animals, BV is most often acquired as an oral infection by infants and juveniles or as a genital infection in sexually mature animals. In many respects the biology of BV is comparable to herpes simplex virus (HSV) in humans and *Cercopithecine herpesvirus* 16 (*Herpesvirus papio* 2; HVP2) in baboons (*Papio spp*.). While there have been no reported cases of human HVP2 infection, BV is well known for its extreme neuropathogenicity when transmitted to non-macaque primates including humans (4, 18, 19). Although zoonotic BV infections are rare, the extreme severity of these infections has resulted in BV being classified as the only biosafety level 4 (BSL-4) herpesvirus. Furthermore, the U.S. government classifies BV as a "select agent" due to concern regarding its potential use as a bioterrorism weapon.

Due to the hazardous nature of BV and the requirement for special biocontainment facilities, relatively little research has been done on BV. An additional difficulty has been the lack of a small animal system which faithfully and reproducibly portrays zoonotic BV infections. Although rabbits are quite sensitive to BV infection, their size, cost, and lack of analytical reagents make this a less than an ideal model system. The development of a small animal model that accurately depicts human BV infections will be of use not only in studying zoonotic B virus infections but also for investigating the more general question regarding zoonoses: what mechanisms cause

some viruses to be so extremely neurovirulent when introduced into a non-natural host species?

Several studies have examined the mouse as a potential small animal model system for studying BV neurovirulence (11, 14). Although very young animals are quite sensitive to BV infection (11), a number of concerns regarding the use of a murine model to study cross-species BV infections have been identified. First, the pathogenicity of different strains of BV in mice varies across the full range of virulence phenotypes from completely apathogenic to extremely neurovirulent (14). Second, the virulence of individual BV isolates in mice is variable depending on the route of inoculation (Ritchey JR, Black DH, and Eberle R; unpublished data). Finally, BV infections in mice are not strictly dose-dependent so that calculation of infectious and lethal doses can be difficult (14). Therefore, while a BV/mouse model may be appropriate for examining certain aspects of BV infections; this system may not be suitable for molecular and genetic analyses aimed at examining the viral mechanisms which determine the outcome of a cross-species BV infection.

In contrast to the wide range of pathogenic phenotypes of BV observed in mice, intra-muscular (i.m.) inoculation of mice with multiple isolates of HVP2 revealed quite a different picture in that all isolates tested fell into one of two distinct subtypes: highly neurovirulent (HVP2nv) or apathogenic (HVP2ap) (16). While all HVP2nv isolates tested produced fulminant, fatal, central nervous system (CNS) infections equivalent to those induced by the most pathogenic strains of BV, HVP2ap-inoculated mice never showed clinical signs of infection (16). In addition, despite the very high degree of

genetic relatedness between BV and HVP2, HVP2 is a BSL-2 pathogen. Thus, all indications are that an HVP2nv/mouse model system may be a safe, consistent, and reproducible model for human BV infections.

Initial experiments with HVP2 in mice used i.m. inoculation to simulate infection via a monkey bite. While a number of human BV infections have resulted from bites, several other routes of transmission have been implicated including eye splashes, scratches from macaques or contaminated equipment, and needle sticks. To fully validate the usefulness of the HVP2nv/mouse system as a model for zoonotic BV infections, it was necessary to assess whether the pathogenic phenotypes observed after i.m. inoculation of mice remained consistent using additional routes of infection. Further, it was important to characterize both the temporal and spatial distribution of HVP2 infection in the mouse CNS. Finally, it was necessary to distinguish between the neuroinvasive and neurovirulent capacity of HVP2ap to determine whether the differences between HVP2nv and HVP2ap were due to the inability of HVP2ap to enter and/or replicate within the CNS.

Methods

Viruses and Cells

Vero cells were cultured in complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM containing 2% FBS. The origin of all HVP2 isolates as well as the purification of viral DNA have been previously described (6-8, 16).

Mouse Inoculations

Female BALB/c mice, weighing 10-12 g (intra-cranial and eye splash inoculation) or 12-14 g (skin scarification) were obtained from Charles River Laboratories (Wilmington, MA, USA). For inoculation, mice were immobilized by anesthetic inhalation (IsoFlo®, Abbott Laboratories, North Chicago, IL, USA). Once infected, mice were observed twice daily for clinical signs of infection. All mice were humanely euthanized by CO_2 inhalation when clinical signs of infection became severe or at termination of the experiment. Sterile PBS was used as diluent for all viral dilutions and as inoculum for negative control mice.

For skin scarification (s.s.), groups of 8 mice were shaved and an 8-mm circle drawn on the left rear flank to identify the inoculation site. A 22-gauge needle was used to superficially scarify the skin inside the circle and 10 µl of inoculum containing ten-fold dilutions of HVP2nv isolate X313 or HVP2ap isolate OU2-5 ranging from 10^2-10^6 PFU was applied to the site with a micro-pipettor.

For eye splash (e.s.) inoculation, 5 µl of inoculum containing 10-fold dilutions of HVP2nv isolate OU1-76 ranging from 10^1-10^6 PFU or HVP2ap isolate OU2-5 ranging from 10^4-10^6 PFU was placed directly into the left eye of mice in groups of five using a micro-pipettor.

For temporal studies, groups of 72 mice were inoculated by s.s. with 10^5 PFU of HVP2nv isolate X313, HVP2ap isolate OU2-5, or diluent as described above. Six mice from each group were euthanized daily 1-9 days post-infection (dpi). Skin from the

inoculation site, sections of lumbar and thoracic spinal cord, and brainstem tissue were harvested from 3 mice in each group and stored at -80 °C. The remaining three mice from each group were processed for histopathological examination.

For intra-cranial (i.c.) inoculation, groups of 5 mice were inoculated using a 50 µl syringe (Model 705LT, Hamilton Company, Reno, NV, USA) with a 27-gauge needle to deliver 10 µl of 10^2-10^6 PFU of HVP2ap isolates A951 or OU2-5, 10^0-10^6 PFU of HVP2nv isolate X313, or diluent into the cerebrum.

Sample Preparation

To obtain template DNA for the real-time PCR assay, total DNA was extracted from tissue samples using the DNAeasy Tissue Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's protocol except that the amount of proteinase K was doubled, DNA was eluted in a 100 µl final volume, and 15 µl of 0.25 % linear polyacrylamide was added to all spinal cord samples prior to ethanol precipitation of the DNA (10). DNA samples were dehydrated in an Eppendorf Vacufuge™ and stored at -20 °C. All DNA samples were resuspended in 10 mM Tris, pH 8.0 (brain and inoculation site epithelial DNA in 200 µl; spinal cord tissue DNA in 35 µl) and quantitated by spectroscopy.

Real-time Quantitative PCR Assay

To avoid potential problems with the real time assay due to subtype-specific sequence variation, HVP2-specific primers and probe were designed from an alignment of the HVP2 UL41 ORF sequence of four HVP2ap isolates and four HVP2nv isolates using the

PRIMEREXPRESS software (Version 2.0, Applied Biosystems, Foster City, CA, USA). The sequence of the forward primer (5'-TGCGCCAACCTCTACCA-3') and reverse primer (5'-TGTCGGTCGTGTGGACGT-3') as well as a TaqMan® MGB probe (5'-CCAACACCGTCGCG-3') labeled at the 5' end with FAM (6-carboxyfluorescein) and at the 3' end with a non-fluorescing quencher, were all designed from the nucleotide alignment of the eight HVP2 sequences. Primers/probes and all real-time PCR reagents, disposables, and equipment were purchased from Applied Biosystems. PCR reactions were performed in a 96-well plate and contained 1x TaqMan® Universal PCR Master Mix, 2.0 µM each primer, 10 nM probe, and 2.0 µl sample DNA in a final volume of 25 µl. The TaqMan® Pre-Designed Assay for 18S rRNA was used as directed by the manufacturer. PCR amplification and detection was performed on an ABI Prism 7000 Sequence Detection System using the following cycling conditions: 1 cycle 50 °C for 2 min, 1 cycle 95 °C for 10 min, and 40 two-step cycles of 95 °C for 15 sec and 60 °C for 1 min. All PCR reactions were carried out in triplicate with appropriate controls run on each plate.

HVP2 DNA and Cellular DNA Standards

For use as a viral standard in the PCR assay, an 800-bp segment of the HVP2 UL41 ORF was amplified by standard PCR using the forward primer HVP2-492 5'-GCATGTTGGAGAAGGCGGAGCTGG-3' and the reverse primer HVP2-493 5'-GACTGGTGCGGAGGGGAGGTTGG-3' (Sigma-Genosys, The Woodlands, TX, USA). The Invitrogen (Carlsbad, CA, USA) TOPO TA Cloning® Kit was used to clone the PCR product and fidelity of the insert confirmed by DNA sequencing. Purified plasmid DNA

was isolated using the QIAGEN Plasmid Maxi Kit and quantitated by spectroscopy. The plasmid standard was used as described on the Applied Biosystems website (*www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf*). The dynamic range for detection of the HVP2 plasmid standard by the real-time PCR assay was 10^0-10^5 viral genome copies (VGC) per reaction.

To correlate 18S rRNA C_T values with mouse cell numbers as a means of normalizing for variable tissue mass, two 25 cm^2 flasks of sub-confluent mouse L929 cells at different cell densities were counted using a hematocytometer. Suspensions containing 1.3 x 10^6 and 3.1 x 10^6 cells were centrifuged at 300 x g for 5 min and each cell pellet resuspended in 500 µl of sterile PBS for DNA extraction. The 18S rRNA assay was used to detect the number of 18S rRNA genes in a 10-fold dilution series of the two DNA samples. The 18S C_T values showed a linear decrease with increasing L cell DNA over a dynamic range of $<10^0$ to $1.3x10^4$ cells. Cellular 18S C_T values were plotted against the number of 18S genes per reaction to generate a standard curve using Microsoft® Excel 2003. Regression analysis was used to calculate the number of mouse cells present in unknown DNA samples based on the 18S C_T values.

Correlation Between Viral Genome Copies and Infectious Virus

To validate the use of real-time PCR for quantifying HVP2 in tissue samples, it was necessary to correlate the number of viral genome copies (VGC) to the number of infectious virions (PFU). Mice in groups of 2 were inoculated by s.s. with 10^5 PFU of HVP2nv isolate OU1-76 or HVP2ap isolate OU2-5. Mice were euthanized at 7 dpi and tissue samples collected from the site of inoculation, spinal cord, brainstem, and spleen.

After the tissue samples were homogenized in 500 µl DMEM with 2% FBS (750 µl for inoculation site skin samples), 450 µl of the liquid was removed, divided equally into two sterile microcentrifuge tubes, and placed at -80 °C. The Qiagen QIAamp 96 DNA Blood Kit was used to extract total DNA from one aliquot for use as template in quantitative real-time PCR assay. The second aliquot was used to quantitate infectious virus using a standard plaque assay (5).

The minimum detection threshold capability for the plaque assay was 2.0×10^1 PFU ml^{-1}. Based on validation of the real-time assay using the viral standard, the minimum detection level of viral genome copies was 5.0×10^2 VGC ml^{-1}. Log values of PFU ml^{-1} were plotted against log values of VGC ml^{-1} and the correlation coefficient calculated using Microsoft® Excel 2003 (Figure 5.1). The correlation coefficient (R= 0.966) demonstrates a strong positive correlation between VGC and PFU in all positive tissue samples. Although there were only two HVP2ap-positive samples, the correlation between VGC and PFU for these samples was the same as for HVP2nv-positive samples. Thus, the sensitivity of the real-time assay was similar regardless of HVP2 subtype. On average, for both HVP2nv and HVP2ap, each PFU correlated to approximately 10^4 VGC.

Histopathology and Immunoassays

Blood was collected by cardiac puncture at the time of death/euthanasia and the serum stored at -80 °C until tested by ELISA to detect anti-HVP2 IgG as described (12, 16). All histological procedures were conducted as previously described (2), (13). For mice inoculated i.c., brains were removed at the time of death/euthanasia and submerged in buffered formalin until dissected for histological examination.

Statistical Analyses

The 50% infectious dose (ID_{50}), 50% clinical disease dose (CD_{50}), 50% CNS disease dose ($CNSD_{50}$) and the 50% lethal dose (LD_{50}) were calculated by probit regression with PROC PROBIT in PC SAS Version 8.2 (SAS Institute, Cary, NC, USA). The values calculated were compared by methods developed for effective dosages (15). The ID_{50} was defined by the presence of serum anti-HVP2 IgG in mice that survived to at least 10 dpi for s.s. experiments or 7 dpi for the i.c. inoculation. Mice that died prior to 10 dpi were assumed to be infected and were included as positives for calculation of ID_{50} values. CD_{50} values were calculated based on mice that developed skin lesions while $CNSD_{50}$ values were based on mice that exhibited clinical signs of CNS disease. LD_{50} values were based on animals that either died as a result of the infection or required euthanasia due to the severity of disease.

Results

HVP2 Pathogenicity Via Skin Scarification

We previously showed that HVP2ap was clinically apathogenic in mice following i.m. inoculation while HVP2nv infection resulted in rapid paralysis and death (16). To determine if the two mouse-defined pathogenic phenotypes were consistent following a different route of inoculation, mice were inoculated via s.s. with HVP2 at doses ranging from 10^2-10^6 PFU. All mice inoculated with 10^6 PFU HVP2nv developed lesions at the inoculation site as well as elsewhere within the dermatome by 4-5 dpi (Figure 5.2A). Mice infected with 10^2-10^5 PFU of HVP2nv also developed lesions at the site of

inoculation with some delay in the appearance of the lesions with lower viral doses (Table 5.1). In contrast, small lesions confined to the site of inoculation were observed 6-7 dpi in mice inoculated with 10^5-10^6 PFU of HVP2ap (Figure 5.2B). These lesions resolved by 9-10 dpi; however, at 12-13 dpi a few of these same mice developed new lesions, primarily at or near the site of inoculation, ipsilateral hind limb, and/or ipsilateral inguinal region. A single mouse inoculated with 10^4 PFU of HVP2ap developed a small lesion at the site of inoculation at 13 dpi. Mice inoculated with doses lower than 10^4 PFU of HVP2ap never showed any clinical signs of disease. Based on these data, the CD_{50} values were determined to be $10^{2.7}$ PFU for HVP2nv and $10^{5.8}$ PFU for HVP2ap.

Based on the presence of serum anti-HVP2 IgG, the ID_{50} value for HVP2ap-infected mice was $10^{3.9}$ PFU (Table 5.1). All HVP2nv-infected mice inoculated by s.s. that exhibited signs of CNS disease succumbed to the infection resulting in identical ID_{50}, $CNSD_{50}$, and LD_{50} values of $10^{2.9}$ PFU. In contrast, $CNSD_{50}$ and LD_{50} values for HVP2ap by s.s. were $>10^6$ PFU, with no HVP2ap-infected mice requiring euthanasia.

Temporal and Spatial Distribution of HVP2 Infection in Mice

Mice were inoculated by s.s. with 10^5 PFU of HVP2 for several purposes: 1) to determine the timeframe required for HVP2nv to enter the CNS, 2) to evaluate the anatomical distribution of HVP2 infection within the mouse CNS, and 3) to quantitate the spread of HVP2nv compared to HVP2ap. At various time points, skin from the inoculation site, lumbar spinal cord, thoracic spinal cord and brainstem were harvested, DNA extracted, and VGC/cell quantitated by real-time PCR. As shown in Figure 5.3A, DNA from both HVP2 subtypes was detected at all dpi from skin at the site of inoculation; however

HVP2nv DNA was present at significantly higher levels than HVP2ap on 4 of 7 dpi. HVP2nv was detected in both the lumbar and thoracic regions of the spinal cord by 4 dpi and 5 dpi respectively, with the amount of virus in both regions increasing between 5-7 dpi (Figure 5.3B and C). In contrast, HVP2ap DNA was not detected in the spinal cord until 5-6 dpi and at significantly lower levels than HVP2nv DNA. Viral DNA was also detected at significant levels in the brainstem of all HVP2nv-infected mice between 6-7 dpi (Figure 5.3D), while HVP2ap was never detected in the brainstem throughout the experiment.

Following s.s. inoculation detectable histological lesions were restricted to the skin, spinal cord and brainstem. By 3 dpi, the epidermis of HVP2nv-infected mice exhibited hyperplasia as well as necrosis of epithelial cells, intranuclear inclusion bodies, and viral antigen immunoreactivity within epithelial cells of the epidermis and hair follicles (Figure 5.4A-B). At 5 dpi, these lesions had progressed to full thickness necrosis of the epidermal and follicular epithelium, formation of serocellular crusts, and intense infiltrates of neutrophilic and mononuclear inflammatory cells within the dermis and subcutis. Evidence of viral infection within the epithelium of HVP2ap-infected mice was evident by 5 dpi and was characterized by occasional scant necrosis of epithelial cells, neutrophilic and mononuclear dermal infiltrate, and the formation of surface crusts; however, full thickness necrosis of the epithelium and herpetic inclusion bodies were not conspicuous. By IHC positive staining was located mostly within the epidermis and hair follicles in HVP2ap-infected mice (Figure 5.4 C-D).

Despite the lack of clinical signs of CNS infection in HVP2ap-infected mice, the development of CNS lesions was similar for the two HVP2 subtypes, differing primarily in the severity of tissue destruction and intensity of viral antigen detected by IHC. In HVP2nv-infected mice, lesions characterized by swelling and necrosis of DRG cells and a mild infiltration by mononuclear inflammatory cells as well as viral antigen were first detected at 4 dpi. In HVP2ap-infected mice a level of involvement comparable to HVP2nv was not seen in the DRG until 6 dpi. While lesions in the DRG of HVP2nv-infected mice progressed with significant inflammation and loss of ganglion cells, DRG lesions in HVP2ap-infected mice did not progress over time. Further, the intensity of IHC staining was always greater in HVP2nv-infected tissues.

At 5 dpi, spinal cord lesions were similar between mice infected with the two HVP2 subtypes, being characterized by mild infiltrates of mononuclear cells within the ipsilateral dorsal funiculus and overlying meninges of the lumbar spinal cord. By day 7 dpi, HVP2nv-infected mice exhibited severe inflammation and spongiosis that in all regions of the thoracic and lumbar spinal cord (Figure 5.4E) while spinal cord lesions in HVP2ap- infected mice (Figure 5.4G) were restricted to the ipsilateral dorsal funiculus of the lumbar and thoracic spinal cord and never developed to the level of severity observed in HVP2nv-infected mice. By IHC, viral antigen distribution correlated with histological lesions, the intensity of viral antigen staining being greater in HVP2nv-infected mice than in HVP2ap-infected animals (Figure 5.4F and H).

Histopathological lesions within the brainstem were seen at 8 dpi in one animal each from the HVP2nv- and HVP2ap-infected groups. While the lesions were nearly

identical in character (Figure 5.4I and K), viral antigen was markedly conspicuous in the HVP2nv-infected mouse but scant in the HVP2ap-infected mouse (Figure 5.4J and L).

HVP2 Pathogenicity Via Intra-cranial Inoculation

Inoculation of high doses of HVP2ap by s.s. resulted in a productive infection, despite a lack of clinical signs of CNS infection, evidenced by both seroconversion and CNS lesions. These data suggested two possible reasons for the apathogenicity of HVP2ap: 1) inefficient viral replication or control of the virus by the host innate immune system at the site of inoculation does not allow generation of sufficient virus for efficient CNS invasion, or 2) HVP2ap is deficient for replication within tissues of the CNS. To test this second possibility, groups of 5 mice were inoculated i.c. with doses of HVP2nv ranging 10^0-10^6 PFU or with 10^2-10^6 PFU of HVP2ap. The ID_{50} value for HVP2nv following i.c. inoculation was < 10^1 PFU while the two HVP2ap isolates had ID_{50} values of $10^{5.1}$ (A951) and $10^{4.3}$ PFU (OU2-5) (Table 5.2). Clinical signs of disease following i.c. inoculation included circling, hyperesthesia, photophobia, and ataxia demonstrated by incoordination and tip-toe walking. All mice that developed clinical signs required euthanasia, so that the $CNSD_{50}$ and LD_{50} value for HVP2nv was ≈ 1 PFU. In contrast, the HVP2ap isolates A951 and OU2-5 had $CNSD_{50}$ and LD_{50} values of $10^{6.1}$ PFU and $10^{5.9}$ PFU, respectively.

Histopathological staining of brains from mice inoculated with HVP2ap revealed that lesions were restricted to a non-suppurative meningitis and subependymal accumulations of granular material that was positive for viral antigen by IHC (Figure 5.5A and B). Apart from these focal deposits that were accompanied by microglia, there

were no inflammatory lesions or distribution of viral antigen distant to the site of inoculation noted within the brain. In contrast, mice that received HVP2nv exhibited inflammation, neuronal necrosis, and viral antigen within the cerebrum as well as regions distant from the inoculation site including the cerebellum and brainstem (Figure 5.5B and C).

HVP2 Pathogenicity Via Eye Splash Inoculation

Mice were inoculated with HVP2 by eye splash to determine if HVP2 could enter the CNS through neural circuits without injury to the eye (corneal scarification), similar to what is thought to have occurred in a case of human BV infection (1). By 3 dpi, all mice inoculated with 10^6 PFU and one mouse inoculated with 10^5 PFU of HVP2nv had pronounced swelling and redness of the conjunctiva in the infected eye. Between 8-11 dpi all mice infected with 10^6 PFU, and one mouse each from the HVP2nv 10^5 and 10^3 PFU groups either died or required euthanasia due to signs of severe CNS disease. Clinical signs of CNS disease were similar to those observed for mice infected i.c. A single mouse inoculated with 10^5 PFU of HVP2ap developed conjunctivitis at 6 dpi similar to that observed in the HVP2nv infected mice. However, the infection resolved by 10 dpi and the mouse remained healthy throughout the experiment.

Discussion

While human BV infections are rare, the high rate of mortality associated with these infections is particularly startling. The availability of a safe, consistent, and reproducible small animal model system amenable to molecular analysis and experimental studies

would greatly facilitate elucidation of viral determinants responsible for the dichotomous behavior of viruses in their natural vs. aberrant host. HVP2nv produces infections in mice which closely parallel human BV infections in many ways and has the added benefit of being a safer, more convenient agent to work with experimentally. This study was undertaken to more fully assess the appropriateness of the HVP2/mouse system as an accurate model of zoonotic BV infections.

One alarming characteristic of human BV infections is that this virus readily invades the CNS regardless of the mode of inoculation. The first set of experiments was designed to evaluate the efficiency of HVP2 infection in mice using epidermal scarification to mimic what occurs during zoonotic transmission of BV via a scratch. For HVP2nv, skin lesions at the site of inoculation were significantly more severe following s.s as compared to i.m., suggesting that HVP2nv replicates more efficiently in the skin than in muscle tissue. Although the LD_{50} value for HVP2nv was similar by both s.s. and i.m. inoculation (16), all HVP2nv-infected mice inoculated with doses as high as 10^5 PFU by s.s. were dead by 8 dpi while 2/8 mice inoculated i.m. with this same dose survived to 11 and 16 dpi and two additional mice survived to termination of the experiment. Thus, inoculation of HVP2nv into the dermis results in more efficient invasion of the CNS and a more severe CNS infection. Weeks et al (17) showed that both HSV-1 and HSV-2 produced more severe primary and secondary lesions following flank scarification as compared to intra-dermal inoculation. Similarly, recent work with *Saimiriine herpesvirus* 1 (a related α-herpesvirus of squirrel monkeys) demonstrated that more severe and consistent infections resulted in mice following epidermal inoculation as compared to i.m. inoculation (3). Epidermal scarification permits efficient viral access to numerous

free sensory nerve endings located above the basement membrane for ascension to neuronal cell bodies in the DRG. Once in the DRG, virus must again replicate and travel back down afferent neurons to the dermatome surrounding the site of inoculation to produce secondary lesions (zosteriform spread) and/or proceed cranially into the CNS. The temporal and spatial distribution of HVP2nv in the mouse CNS as well as the appearance of skin lesions at the site of inoculation which correlated with a rapid onset of CNS signs and death are both consistent with this scenario.

One interesting observation was the "all-or-none" infection process in mice inoculated s.s. with HVP2nv: mice either developed a rapidly fatal CNS infection prior the appearance of an HVP2-specific IgG response or they did not become infected as evidenced by a lack of seroconversion in surviving mice. The single exception was one mouse in the 10^2 PFU dosage group that survived to 14 dpi and was seropositive at death. Experiments where virus was inoculated directly into the brain produced comparable results, indicating that once HVP2nv invades the nervous system the infection inexorably progresses to death. In contrast, both mice that survived i.m. inoculation with 10^5 PFU of HVP2nv displayed mild CNS signs which resolved over the course of the experiment, and others that survived at least 10 dpi were seropositive, even those that did not display clinical signs of CNS infection. Following i.m. inoculation, virus deposited into muscle tissue would be able to elicit an immune response, even if it was unable to gain entry into the CNS to cause overt disease. Since s.s. inoculation gives the virus direct access to free sensory nerve endings present in the dermis, it is easier for the virus to enter into these sensory nerves for transport to the DRG and entry into the CNS. If the virus is unable to gain entry into the nerves for some reason (i.e. low inoculum dose), the virus may be

controlled by the innate immune system such that a specific immune response is not induced and no clinical disease develops. If in humans BV also either invades the nervous system resulting in an extremely severe and generally fatal infection, or does not successfully enter the nervous system and is controlled by a local immune response, this could explain the lack of any concrete evidence of asymptomatic BV infections in humans (9).

Although most human BV infections have been attributed to macaque bites or scratches, needle sticks, or abrasive contact with contaminated fomites, there is a single reported case of a human infection resulting from contaminated fluid entering the CNS through an eye splash (1). The results of the HVP2 eye splash inoculation of mice demonstrate that while HVP2nv replicates within the eye, only very high doses consistently produce a severe, fatal CNS infection. The relative inefficiency of HVP2nv entry into the CNS through an uninjured eye may reflect a similar situation in human BV infections, thus explaining the dearth of documented cases attributed to an eye splash. Further, while the eye splash is an ineffective route of entry for HVP2 into the CNS, the fact that only HVP2nv caused CNS disease further strengthens the conclusion that the pathogenic phenotypes of the two HVP2 subtypes are consistent regardless of route of inoculation.

One distinct asset of the HVP2nv/mouse system is the existence of HVP2ap isolates that provide a "ready to use" naturally occurring apathogenic form of HVP2nv. The fact that the majority of HVP2 isolates characterized to date represent the HVP2nv subtype suggests that HVP2nv represents the wild-type while HVP2ap is a somewhat less

successful mutant. As a first step in determining how HVP2nv and HVP2ap differ within the mouse model, the temporal progression of HVP2ap was compared to HVP2nv following s.s. inoculation of mice. Regardless of the peripheral replication of HVP2ap at the site of inoculation and its ability to invade the CNS, no clinical signs of CNS disease were noted in any HVP2ap-infected mice following s.s. infection with doses as high as 10^6 PFU. In addition, IHC staining of brain tissue samples from mice inoculated i.c. with HVP2ap revealed that HVP2ap was effectively sequestered at the site of inoculation, suggesting that HVP2ap is not competent for spread within the brain. These results suggest that a lack of neurovirulence and not a lack of neuroinvasiveness is a major reason for the differences observed between HVP2ap and HVP2nv in mice. This information will be useful in discerning which viral genes differ between the two HVP2 subtypes and may account for the dichotomous pathogenicity in mice.

In conclusion, HVP2nv appears to behave in mice very similar to BV in humans. Irrespective of the route of inoculation, HVP2nv readily invades the CNS and produces a fulminant ascending encephalomyelitis which proves fatal once virus reaches the brainstem. This neuropathogenic behavior is observed for all HVP2nv strains tested, and thus appears to be an inherent property of the virus and not peculiar to a single isolate. The degree of genetic relatedness between HVP2 and BV and their biological similarities (pathogenicity in mice, resistance to anti-HSV drugs, *in vitro* replication, etc.) coupled with a preponderance of evidence demonstrating just how closely HVP2 infection in mice parallels human BV infections all support the appropriateness of the HVP2nv/mouse system as a model for investigating zoonotic BV infections.

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Table 5.1

HVP2 Pathogenesis in Mice Following Inoculation by Skin Scarification

Virus	Dose (PFU)	No. mice that developed: Skin Lesions	CNS Signs	Death	ELISA OD Values †	Seropositive‡
HVP2nv						
	10^6	7/7	7/7	7/7 (6 dpi)*		NSA
	10^5	8/8	8/8	8/8 (6 dpi)		NSA
	10^4	8/8	8/8	8/8 (7 dpi)		NSA
	10^3	5/8	4/8	4/8 (8.25 dpi)		0/4
	10^2	1/8	1/8	1/8 (14 dpi)	0.403	1/8
HVP2ap						
	10^6	5/8	0/8	0/8	0.382 (0.235-0.513)	8/8
	10^5	1/8	0/8	0/8	0.321 (0.154-0.558)	8/8
	10^4	1/8	0/8	0/8	0.436 (0.170-0.594)	6/8
	10^3	0/8	0/8	0/8		0/8
	10^2	0/8	0/8	0/8		0/8

*Average time of death for each group is shown in parentheses.

† The mean OD values for positive sera (positive cutoff ≥ 0.100) for anti-HVP2 IgG in serum from mice collected 10-19 dpi. Numbers in parentheses are the range of OD^{490} values of positive sera.

‡ Number of positive sera/total number of sera tested. Sera were not tested for mice that died prior to 10 dpi. NSA, no sera available for testing.

Table 5.2

HVP2 Pathogenesis in Mice Following Intra-Cranial Inoculation

Virus	Dose (PFU)	Death[*]	Mean Survival Time (dpi)[†]	ELISA OD Values[‡]	ELISA Seropositive[§]
HVP2nv					
	10^6	4/4[‖]	1.39 (1.0 - 2.0)	NSA	NSA
	10^5	5/5	1.42 (1.27-2.0)	NSA	NSA
	10^4	5/5	4.20 (4.0 – 5.0)	NSA	NSA
	10^3	5/5	5.60 (5.0 – 7.0)	NSA	NSA
	10^2	5/5	5.80 (5.0 – 6.0)	NSA	NSA
	10^1	4/5	6.75 (6.0 – 7.0)	0.283 (0.228 – 0.340)	4/5
	10^0	2/5	8.50 (6.0 – 11.0)	0.641 (0.151 – 1.130)	2/3[¶]
HVP2ap					
OU2-5	10^6	4/5	1.45 (1.27 – 2.0)	NSA	NSA
	10^5	0/5	ND	0.142 (0.112-0.168)	4/5
	10^4	0/5	ND	0.186 (0.140-0.231)	2/5
	10^3	0/5	ND		0/5
	10^2	0/5	ND		0/5
A951	10^6	1/5	2.00	0.140	4/4
	10^5	0/4	ND	0.113	¼
	10^4	0/5	ND		0/5
	10^3	0/5	ND		0/5
	10^2	0/5	ND		0/5

[*] Number of mice in each group that died or were humanely euthanized prior to 14 dpi. ND, no death.
[†] The mean survival time for groups of mice following i.c. inoculation. Numbers in parentheses represent the range of survival times for mice in each group.
[‡] The mean OD values for positive sera (positive/negative cutoff ≥ 0.100) for anti-HVP2 IgG in serum from mice collected 7-19 dpi. Numbers in parentheses are the range of OD values of positive sera. Sera were not tested for mice that died at < 7 DPI. NSA, no sera available for testing.
[§] Number of positive sera/total number of sera tested
[‖] One mouse survived to 7 dpi. The large degree of variance from the group mean time of death suggested experimental error and the mouse was dropped from the experiment.
[¶] Both mice that died were found dead in the morning; serum was available only from the three survivors.

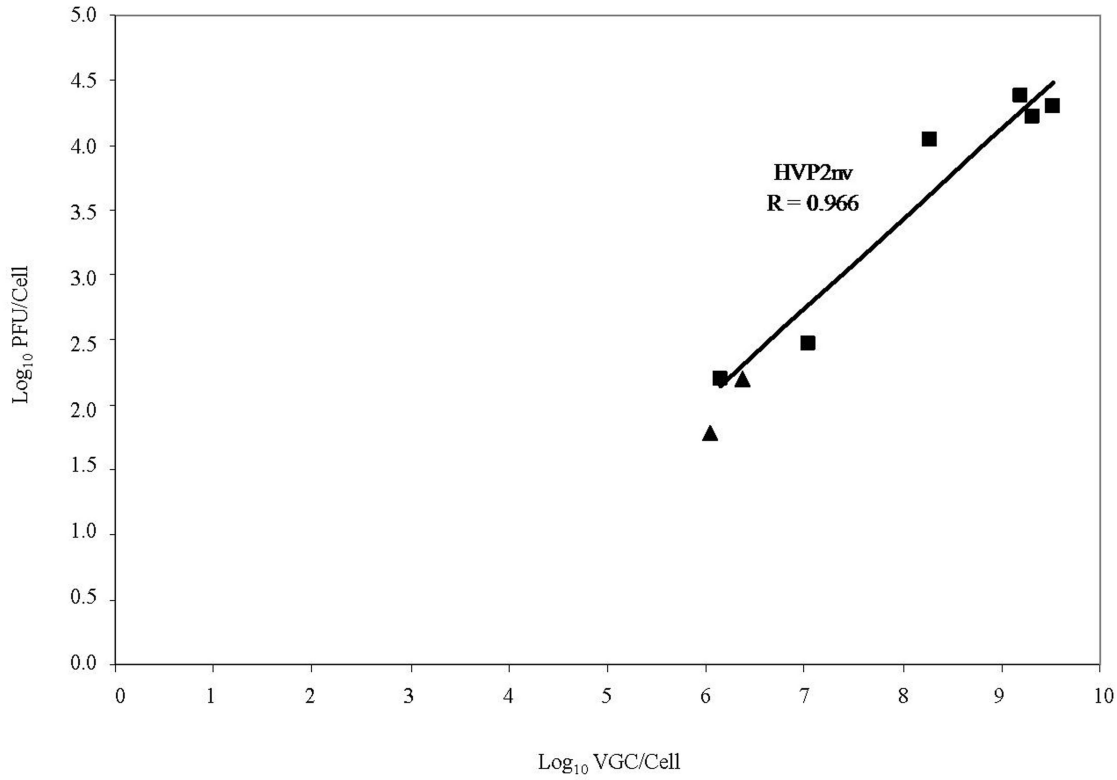

Figure 5.1 - Correlation of viral genome copy (VGC) to plaque forming units (PFU). Tissue samples representing skin, spinal cord, spleen and brainstem from two HVP2nv- (■) and two HVP2ap-infected (▲) mice were assayed for VGC by real-time PCR and PFU values were determined by plaque assay. Each data point represents the average VGC ml^{-1} value for triplicate real-time PCR reactions and PFU ml^{-1} for duplicate plaque assays. Although there were only two HVP2ap-positive samples, the correlation between VGC and PFU for these samples was the same as for HVP2nv-positive samples. The correlation coefficient of 0.966 demonstrates a strong positive correlation between VGC and PFU for both HVP2 subtypes in infected mouse tissues.

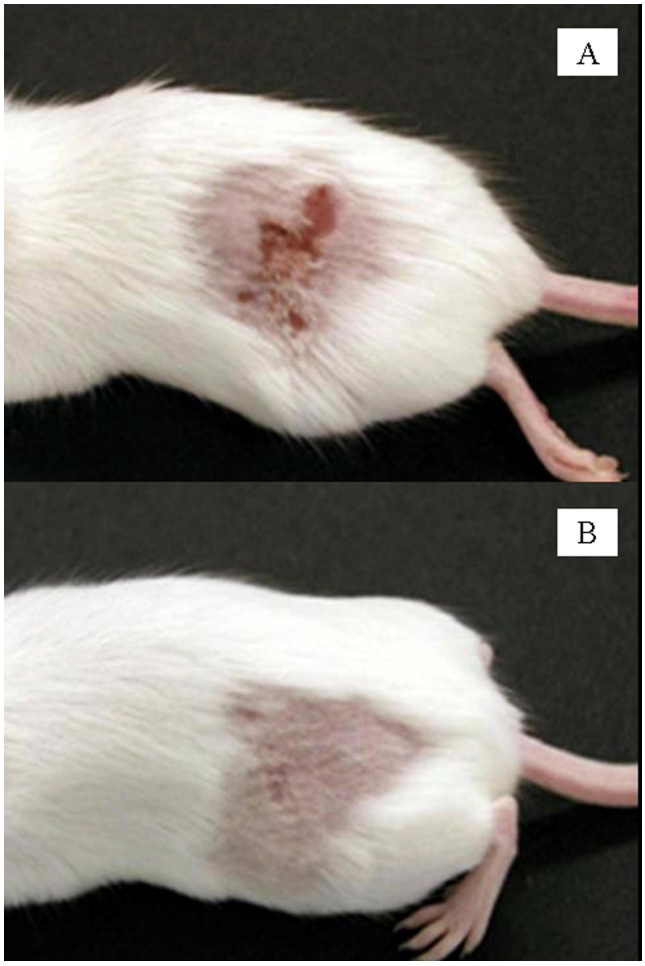

Figure 5.2 - Skin lesions at 6 dpi in mice infected by s.s. with 10^6 PFU of HVP2. In addition to more severe lesions at the site of inoculation, HVP2nv-infected (A) mice displayed ipsilateral flaccid hind limb paralysis by 6 dpi which was not noted in any HVP2ap-infected mice (B) throughout the experiment.

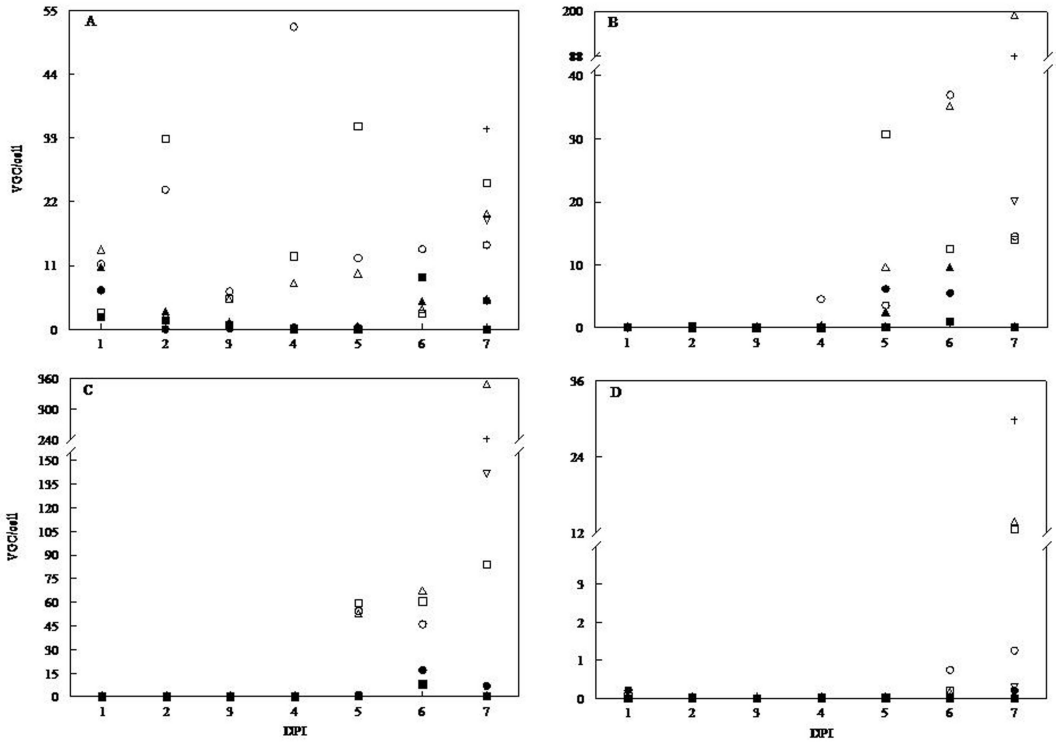

Figure 5.3 - Real time PCR quantitation of viral genome copies (VGC) cell^{-1} in HVP2-infected mouse tissues 1-7 dpi. Filled symbols (■,▲,●) represent individual HVP2ap-infected mice and open symbols (□,Δ,○,+,▽) identify individual HVP2nv-infected mice. Tissues examined were skin from the site of inoculation (A), lumbar spinal cord (B), thoracic spinal cord (C), and brainstem (D).

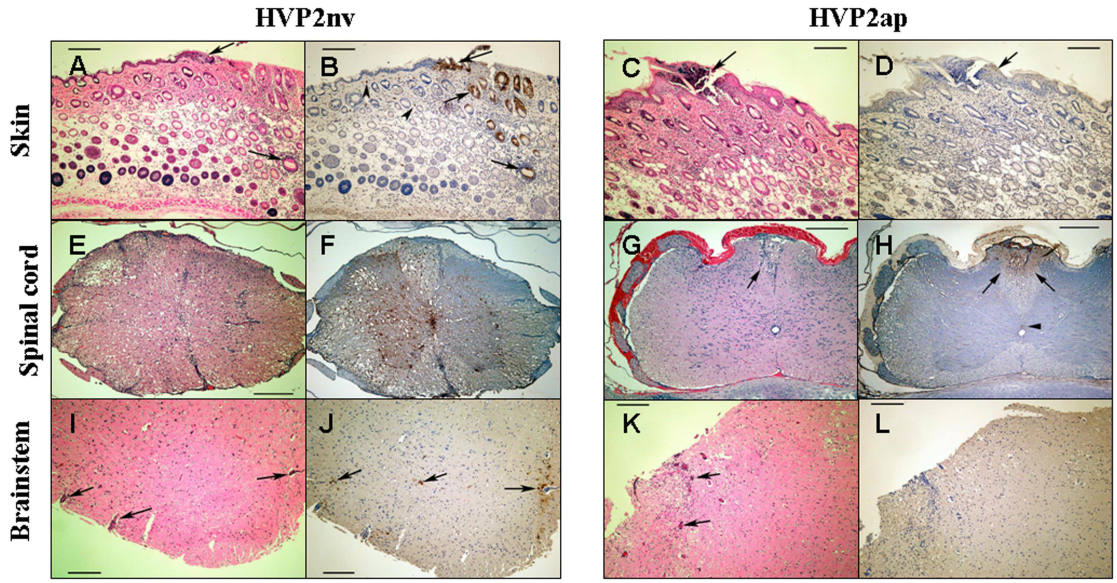

Figure 5.4 - Histological lesions present in mice infected with HVP2.

Skin: HVP2nv-infected mice exhibited epidermal hyperplasia and necrosis (arrowhead, A), dermatitis, and folliculitis (arrow, A). Viral antigen was conspicuous within epidermal and follicular epithelial cells (arrows, B). HVP2ap-infected mice showed focal effacement of the epidermis at the scarification site (arrow, C) with scant viral antigen predominantly in epidermal epithelial cells (arrow, D).

Spinal cord: HVP2nv-infected mice exhibited severe inflammation, spongiosis, and necrosis in both ipsilateral and contralateral regions of the spinal cord (E) accompanied by widely distributed viral antigen by IHC (F). In HVP2ap-infected mice lesions were restricted to mild mononuclear inflammation and subtle spongiosis of the ipsilateral dorsal funiculus (arrow, G). Viral antigen detected by IHC was scant (arrows, H).

Brainstem: Lesions consisting primarily of perivascular cuffing (arrows, I & K) by mononuclear inflammatory cells were present in one mouse each (8 dpi) from the

HVP2nv- (I) and HVP2ap-infected (K) mice. By IHC staining, viral antigen was distributed throughout the brainstem of HVP2nv-infected mice (arrows, J), but was not detectable in the brainstem of HVP2ap infected mice (L). H&E stain (Panels A, C, E, G, I, K). IHC with Mayer's haemotoxylin counterstain (Panels B, D, F, H, J, L). Bar ≈ 180 μm (Panels A-D, I-L) and 250 μm (Panels E-H).

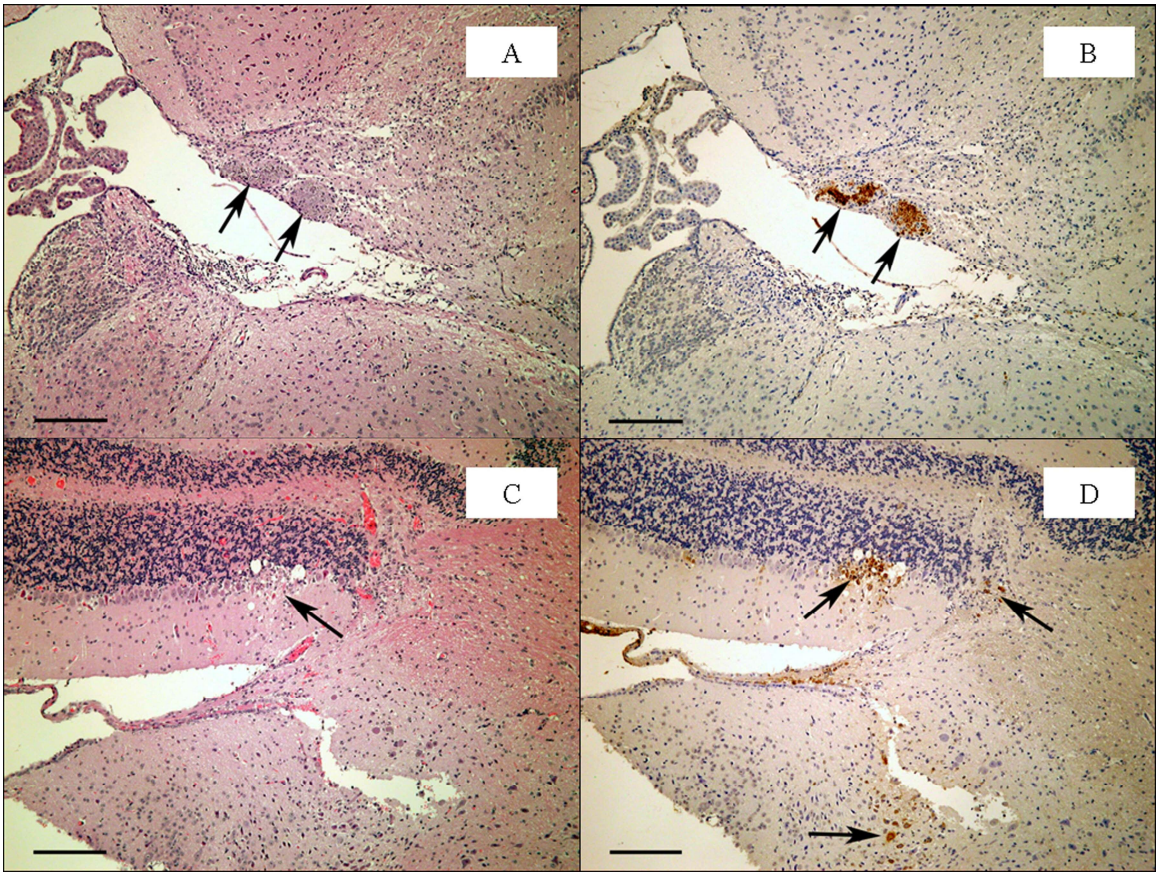

Figure 5.5 - Brain tissue from mice inoculated i.c. with HVP2. Lesions in HVP2ap-infected mice included a mild to moderate non-suppurative meningitis and subependymal deposits of granular basophilic material (arrows, A) that were positive for viral antigen by IHC (arrows, B). HVP2nv-infected mice exhibited evidence of dissemination of viral infection characterized by inflammation, necrosis and viral antigen distributed as far as the cerebellum and brainstem. In the cerebellum, the Purkinje cells were especially affected by necrosis (arrows, C) with confirmation of viral antigen by IHC (arrows, D). H&E stain (A&C). IHC stain (B&D) with Mayer's haematoxylin counterstain; Bar = 180µm.

CHAPTER VI

PRIMARY MOUSE DERMAL FIBROBLAST CELL CULTURES AS AN IN VITRO MODEL SYSTEM FOR THE DIFFERENTIAL PATHOGENICITY OF CROSS-SPECIES *Herpesvirus papio* 2 INFECTIONS

Summary

Infection of mice with *Herpesvirus papio* 2 (HVP2) parallels zoonotic monkey B virus infections. A major benefit of the HVP2/mouse model is the existence of two HVP2 subtypes: HVP2nv rapidly invades and destroys the CNS while HVP2ap produces no clinical signs and mild histopathological lesions. However, in the natural baboon host no difference in pathogenicity of HVP2 subtypes is evident. Primary dermal fibroblast cells were evaluated as a model system for defining virus-host interactions that influence the outcome of a cross-species infection. No differences in plaque formation or virus replication were observed between HVP2 subtypes in primary baboon dermal fibroblast cultures. In contrast, when primary mouse dermal fibroblasts (PMDF) were infected, HVP2nv replicated to higher titers and was more efficient at shutting down host-cell protein synthesis compared to HVP2ap. HVP2ap-infected PMDF cells produced more IFN-β compared to HVP2nv, and IFN-β pretreatment of PMDF cultures inhibited HVP2ap replication but did not affect HVP2nv. The differential pathogenicity of HVP2 subtypes in mice and the lack of such differences in the natural baboon host are recapitulated in the primary dermal fibroblast cell culture system. This model may prove

useful in examining early, local, host responses that influence the outcome of cross-species infections.

Introduction

In contrast to the relatively mild, self-limiting infections that occur in the natural host, when some herpesviruses infect a non-natural host they produce very severe and frequently fatal infections involving the central nervous system (CNS). *Cercopithecine herpesvirus* 1 (monkey B virus; BV) is an alpha-herpesvirus indigenous to macaques that is clinically similar to herpes simplex virus (HSV) in humans [6, 10, 11, 13, 23, 24]. However, when transmitted to humans or other non-macaque primates, BV invades peripheral nerves, ascends into the CNS and causes a severe and usually fatal encephalomyelitis [10, 14, 21,24]. A thorough understanding of the viral and host mechanisms that interact to determine the outcome of cross-species or zoonotic infections is critical to controlling and treating such infections.

The baboon herpesvirus *Cercopithecine herpesvirus* 16 (*Herpesvirus papio* 2; HVP2) is very closely related to BV [5, 12]. Although many humans have been bitten and scratched by baboons, there are no reported incidents of HVP2 infection or death in humans. However, in BALB/c mice one subtype of HVP2 (HVP2nv) produces a fulminant, rapidly fatal CNS infection. A second subtype (HVP2ap) produces no clinical signs of disease and infection results in only minimal tissue destruction at both the site of inoculation and within both the peripheral and central nervous system [14, 15, 17].

Recent work demonstrated that the clinicopathogenesis of HVP2nv in mice closely parallels what has been observed in cases of human BV infection [17]. Furthermore, as for BV in macaques, HVP2 subtypes are indistinguishable in the natural baboon host with regard to their pathogenesis as well as the immunological response they evoke [16]. The HVP2/mouse system thus represents an ideal model system for investigating virus-host interactions responsible for the extreme neurovirulence of alpha-herpesviruses in cross-species or zoonotic infections. Having a parallel *in vitro* model system that similarly differentiates the two HVP2 subtypes would make this mouse/baboon-HVP2 model system much more amenable to molecular dissection of the viral and host mechanisms underlying species-specific pathogenesis.

This study describes the use of primary mouse (PMDF) and baboon (PBDF) dermal fibroblast cell cultures as an *in vitro* model system for investigating virus-host interactions that occur at the site of infection. It is the sum of these interactions that influences the ultimate success or failure of the virus to establish a productive infection in a non-natural host species as compared to what occurs in the natural host where infections are effectively controlled by the host immune response.

Materials and Methods

Cell Cultures

PMDF cell cultures were prepared from 10-12 g BALB/c mice (Charles River Laboratories; Wilmington, MA, USA) and PBDF cultures from a stillborn baboon infant basically as described [1]. The skin was shaved, disinfected with ethanol, and a section

(6 x 6 cm baboon and 4 x 4 cm mouse) removed from the abdomen taking care to separate the skin from the internal membrane. After washing with sterile PBS, skin sections were cut into smaller pieces and treated with 0.25% trypsin at 37 ^{o}C. After 15 minutes, the liquid was removed to a 50 ml centrifuge tube with 5 ml fetal bovine serum (FBS), 10 ml culture medium containing 50 U/ml penicillin/streptomycin (Mediatech, Inc., Herndon, VA) and 2.5 μg/ml Fungizone® (Invitrogen, Carlsbad, CA). The trypsin treatment was repeated a total of four times. After each treatment, the liquid was pooled in a 50 ml centrifuge tube and kept on ice. As a final chemical dissociation step, the intact skin sections were treated with 200 U/ml of collagenase (Sigma, St. Louis, MO) for 20 minutes at 37 ^{o}C. Finally, the remaining undigested tissue was pushed through a sterile screen using the plunger from a 10 ml syringe to physically disrupt the remaining cells. Pooled cells were centrifuged for 10 minutes at 100 x g at 4 ^{o}C to remove trypsin before seeding cells in two (PMDF) or three (PBDF) 100 mm^2 tissue culture plates with 20 ml culture media plus antibiotics and Fungizone®.

Once established, all primary cell cultures were maintained in complete Dulbecco's modified Eagle medium (DMEM) containing 15% FBS plus 4 mM L-glutamine (without antibiotics or Fungizone®) and propagated at 37 °C. Cells were split at a ratio of 1:3 for experiments. Once infected, cells were maintained in DMEM containing 2% FBS. Infected PMDF cultures were initially incubated at the standard 37 °C and also at 39 °C to more closely approximate the normal body temperature of mice [18]. However, once 39 ºC was determined to be the optimum incubation temperature, infected PMDF cultures were incubated at 39 ºC in all subsequent investigations. PBDF cultures were incubated at 37 °C following infection (normal baboon body temperature).

To ascertain the lifespan of PMDF cell cultures, 24-well trays of confluent PMDF cells at passages 1-6 were infected with multiple isolates representing both HVP2 subtypes at a multiplicity of infection (MOI) of 0.4 PFU/cell. Infected cells were harvested at 3 days post-infection (PI) and infectious virus titrated by plaque assay. The production of infectious virus and the differential sensitivity of the PMDF cells to the HVP2 subtypes did not fluctuate across passages 1-6 of the cells (Figure 6.1). By passage 7, the morphology of the cell population began to change and the rate of cell division slowed. Thus, PMDF cells were not viable for experimental use beyond passage six. No differences in the susceptibility to HVP2ap or HVP2nv were noted when PMDF cells were recovered after storage in liquid nitrogen (data not shown). These results indicate that the sensitivity of PMDF cultures to HVP2 does not vary during limited passage of the cells.

Viral Assays

The origins and isolation of all HVP2 isolates used in these experiments have been previously described [5, 7, 15]. In these studies, four HVP2 isolates were used as reference strains: OU2-5 and A951 (HVP2ap) and OU1-76 and X313 (HVP2nv). Additional strains were used to assess viability of PMDF cells over multiple passages: OU4-8 (HVP2ap) and A189164 (HVP2nv). Infected cells were photographed using a Nikon Eclipse TE-200 inverted fluorescent microscope (Melville, NY) with attached RS Photometrics digital camera. One-step growth curves were performed using confluent PMDF cell monolayers in 24-well trays. Cells were infected at an MOI of 20 PFU/cell. After 1 hour at 39 ºC the inoculum was removed, cells were washed twice with sterile

PBS, and 0.5 ml fresh maintenance medium added. The plates were incubated at 39 °C and infected cells harvested from duplicate wells at designated time points as previously described [15].

Infected Cell Protein Synthesis

Confluent PMDF cultures in 12-well trays were infected at an MOI of 4.0 PFU/cell and pulse-labeled with 30 µCi/ml of ^{35}S-Met/Cys for one hour intervals from 1-12 hours PI. Cells were scraped into the medium, pelleted by centrifugation, resuspended in water and frozen at -20 ºC. For SDS-PAGE, samples were diluted 1:2 in loading buffer and heated at 98 ºC for 3 minutes. Proteins were separated by SDS-PAGE on a 10% gel as previously described [4, 9].

IFN-β Assays

Cells were infected at an MOI of 1.2 PFU/cell with HVP2ap or HVP2nv. Media was collected, clarified by centrifugation, and IFN-β levels quantitated using mouse IFN-β ELISA kit (R&D Systems, Minneapolis, MN). Where PMDF cell cultures were pretreated with IFN-β, cells were grown in 24-well trays and recombinant murine IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) added to the media. After 18 hours, the IFN-β medium was removed and cells were infected. Infected cells were harvested at 24 hours PI and infectious virus quantitated by standard plaque assay.

Results

To determine if the differential pathogenicity of HVP2 subtypes could be observed in the cell culture model, confluent PMDF cells were infected with 10-fold dilutions of several isolates of HVP2nv or HVP2ap ranging from 0.04 - 4.0 PFU/cell. At an MOI of 4.0 PFU/cell all HVP2 strains destroyed the PMDF cell monolayer by 24 hours PI. This MOI would have infected most cells in the culture so that multiple rounds of viral replication were not necessary to destroy the cell monolayer. Figure 6.2 compares HVP2nv- and HVP2ap-infected cultures at multiple time points following infection with 0.04 PFU/cell, an MOI which produced individual plaques. While plaques had developed in both HVP2nv- and HVP2ap-infected cells by 24 hours PI, HVP2nv plaques continued to expand so that by 2 days PI the entire cell monolayer was destroyed. However, after 24 hours PI HVP2ap plaques ceased to increase in size and uninfected cells began to fill in the plaques such that by 3 days PI the monolayer was once again confluent, although rounded infected cells remained visible embedded within the monolayer. In contrast to the differential replication of HVP2 subtypes in PMDF cells, all HVP2 isolates replicated with equal efficiency in PBDF cell cultures, producing 4+ CPE by 2 days PI at an MOI of 0.04 PFU/cell.

To confirm the apparent disparate replication of HVP2 subtypes in PMDF cells as well as the differences observed between HVP2-infected PMDF vs. PBDF cell cultures, 12-well trays of baboon or mouse fibroblast cell cultures were infected at an MOI of 0.04 PFU/cell with two strains each of HVP2ap and HVP2nv. At 2 days PI, infectious virus was quantitated by standard plaque assay. As shown in Figure 6.3, all HVP2 isolates

replicated to titers exceeding 10^7 PFU/ml in PBDF cell cultures and titers of infectious virus were less in PMDF cells than in PBDF cells for all HVP2 isolates. This finding is consistent with what was observed for HVP2 replication in Vero vs. mouse L cells; regardless of subtype, HVP2 replicates to higher titers in primate cell lines than in murine cells [15]. However, in PMDF cell cultures HVP2nv produced over 100-fold more infectious virus than HVP2ap. Thus, the PMDF culture model system distinguished between HVP2 subtypes based on the host cell species and not the type of cell line (i.e. primary vs. continuous).

To assess the ability of the two HVP2 subtypes to replicate in PMDF cells, one-step growth curves were performed. PMDF cell cultures were infected using an MOI of 20 PFU/cell to ensure that all cells were infected. As shown in Figure 6.4, there was no difference in the amount of infectious virus produced or the kinetics of virus production by the two HVP2 subtypes. By 20 hours PI, both HVP2 subtypes had completely destroyed the cell monolayers. This indicates that the difference between the two HVP2 subtypes that affects their ability to replicate in PMDF cells can be overcome by using a high MOI.

The fact that the PMDF cell model seemed to accurately represents the differential pathogenicity of the HVP2 subtypes *in vivo* led to two important hypotheses: 1) the factor(s) which limits the spread of HVP2ap acts very early after infection, and 2) the host cell factors involved in determining the outcome of the infection must be present in PMDF cultures and are able to act without the involvement of other factors that need to be recruited to the site of infection *in vivo* (e.g. natural killer cells). One possible

explanation is that something occurs following infection of individual cells that induces an anti-viral response and renders uninfected neighboring cells in the culture resistant to infection. Since production of interferon (IFN) is an initial response of infected cells and a critical initiating factor of the innate antiviral immune response, we examined IFN-β production in PMDF cultures following infection. PMDF cultures were infected with an MOI 1.2 PFU/cell with HVP2ap or HVP2nv and IFN-β in the media quantitated by ELISA. At 8 hours PI, no IFN-β was detected (Figure 6.5). HVP2nv-infected PMDF cultures produced minimal levels of IFN-β at 16 hours PI and levels increased only slightly by 24 hours PI. In contrast, HVP2ap-infected cells produced high levels of IFN-β as early as 16 hours PI with the level of IFN-β present in the extracellular media increasing substantially by 24 hours PI. These results suggest that one difference between HVP2 subtypes may be the ability of HVP2nv to either prevent expression of IFN-β or subvert the anti-viral effects of the cytokine.

If HVP2ap is less efficient at shutting down host cell protein synthesis and initiating viral protein synthesis, it would not only lag behind in the production of infectious virus but also be subjected to more host cell responses designed to control the viral infection and protect bystander cells. To investigate this possibility, PMDF cultures were infected with HVP2nv or HVP2ap at an MOI of 4.0 PFU/cell and pulse-labeled with ^{35}S-Met/Cys at one hour intervals. Infected cell proteins were examined by SDS-PAGE (Figure 6.6). While dramatic differences were not apparent between HVP2nv- and HVP2ap-infected cells, more subtle differences were evident. First, HVP2nv appeared to shut down host cell protein synthesis earlier than did HVP2ap (Figure 6.6; 2-3 hours PI). Secondly, viral proteins were evident earlier in HVP2nv infected cells than

in HVP2ap-infected cells (Fig. 6.6; 1-2 hours PI) and by 3-4 hours PI HVP2nv viral protein expression was higher than HVP2ap levels. Due to the high MOI, by 8-10 hours most cells in the cultures had been destroyed so that the level of protein synthesis decreased significantly. Thus, it appears that HVP2nv is more efficient than HVP2ap at taking over the host cell protein synthesis machinery for production of viral proteins.

To determine the sensitivity of both HVP2 subtypes to the anti-viral effects of IFN-β, PMDF cell cultures were pretreated with 0, 25, 100 or 500 U/ml recombinant murine IFN-β. After 18 h, the IFN-β medium was removed and cells were infected with HVP2nv or HVP2ap at an MOI of 4.0 or 0.04 PFU/cell. Infected cells were harvested at 24 hours PI for titration of infectious virus. At an MOI of 4.0 PFU/cell, pre-treatment of PMDF cell cultures with IFN-β did not significantly affect the production of infectious virus for either HVP2 subtype (Figure 6.7A). However, at the lower MOI HVP2nv replication remained unaffected while the titer of HVP2ap was reduced approximately 100-fold by the addition of all concentrations of exogenous IFN-β (Figure 6.7B). These results demonstrate that HVP2nv is not susceptible to the inhibitory effects of IFN-β, being able to overcome the anti-viral state induced in bystander cells.

Discussion

Previous attempts to develop an *in vitro* system for distinguishing between the two HVP2 subtypes were unsuccessful. Despite the dichotomous neurovirulence of the two HVP2 subtypes in mice, they exhibit no difference in their ability to replicate in a wide variety of primate and mouse tissue culture cell lines including commercially available mouse embryo fibroblasts [16, unpublished]. While it is common among herpesviruses that a

defect *in vivo* is not necessarily reflected *in vitro*, a cell model system that accurately reflects the variable pathogenic phenotypes of the HVP2 subtypes observed *in vivo* would be a tremendous asset in dissecting out the early, local, virus-host interactions that account for the dissimilar pathogenicity of the two HVP2 subtypes in a cross-species infection.

Infection of primary cell cultures used to represent the site of inoculation in both the natural (PBDF) and non-natural (PMDF) host species confirmed that the PMDF cell culture model system distinguishes between HVP2 subtypes based on an inherent property of the virus that affects how the virus interacts with the host cell in cross-species infections. The lack of discernable differences between HVP2 subtypes in PBDF cell cultures indicates that a very different interaction must take place between the virus and cells of the natural host species.

The ability of both HVP2 subtypes to produce equal amounts of infectious virus when PMDF cells are infected at an MOI sufficient to infect most cells in the culture suggests that HVP2ap is capable of replicating almost as well as HVP2nv during the initial round of replication. However, SDS-PAGE analysis of infected cells demonstrated that slight differences are evident in the initial round of replication. It is the magnification of these differences over subsequent rounds of replication that produce the differential replicative phenotypes of HVP2 subtypes. Consistent with this, when most of the cells are not initially infected, something occurs in PMDF cultures which adversely affect the ability of HVP2ap to undergo subsequent rounds of replication, suggesting that there is in fact a defect in the ability of HVP2ap strains to sustain replication in PMDFs.

Since high MOI infection can overcome this deficit, the functional deficiency in HVP2ap appears to be a subtle one, its effect only being evident over multiple rounds of viral replication.

The ability of HVP2ap to initially replicate in PMDF cultures at a level equal to HVP2nv but to then produce decreasing amounts of infectious virus over successive rounds of replication corresponds with the initial formation of plaques with subsequent "healing" of the cell monolayer as time progresses. Further, the inability of HVP2ap to sustain infection in PMDF cultures reflects what is seen *in vivo*: while HVP2ap is able to replicate and spread, it does so very inefficiently as evidenced by the mild skin lesions at the site of inoculation, the paucity of viral antigen in lesions, the minimal amount of viral DNA present in tissues, and the inferior immune response generated by HVP2ap as compared to HVP2nv [15, 17].

The typical process of α-herpesvirus infection includes local replication at the site of inoculation followed by spread into the CNS, usually via sensory afferents [20]. From studies on HSV, it appears that the amount of virus present at the peripheral site is directly related to the ability of the virus to enter sensory neurons and thus invade the CNS [25]. Previous results demonstrated that compared to HVP2nv, isolates of HVP2ap replicate less efficiently in mouse skin at the site of inoculation followed by a temporal delay in neuroinvasion coupled with poor replication efficiency within tissues of the nervous system [15, 17]. This suggests that the block in HVP2ap infection occurs at the site of inoculation very early after infection. As the mechanisms of innate immunity are responsible for early and rapid control of both viral replication and spread, the innate

immune response is likely critical in determining the pathogenic phenotype and outcome of HVP2 infection in a mice. However, the disparity between the replication HVP2nv and HVP2ap *in vitro* precludes involvement of any innate immune factors which must be recruited to the site of infection. Rather, the critical factor in determining the replicative ability of HVP2 subtypes exists and is active within the confines of the PMDF cell culture model.

The antiviral effects of IFN-β provide extremely rapid and effective control of viral replication at the site of initial infection. Production of IFN-β is the first response due to constitutive expression of IFN regulatory factor 3 (IRF3) in fibroblasts and epithelial cells which are the cells initially infected by herpesviruses [8, 19]. The fact that HVP2nv induces only minimal amounts of IFN-β compared to HVP2ap raises several interesting possibilities. One option is that the low levels of IFN-β produced following HVP2nv infection result from the increased proficiency of HVP2nv at shutting down host cell protein synthesis as observed in the radiolabeling experiments. However, if an overall decrease in host cell protein synthesis accounted in full for the disparate IFN-β levels, one would not expect the differential sensitivity of HVP2 subtypes to exogenous IFN-β that was observed in PMDF cell cultures. That HVP2nv replication is not inhibited by pretreatment of cells with IFN-β indicates that this virus can overcome the anti-viral state induced in uninfected cells by IFN-β.

As is common for α-herpesviruses, HVP2 has several genes which abrogate the antiviral effects of IFN through interference with either its production or function: US11, UL41 (virion host shutoff; *vhs*), and RL2 (infected cell protein 0; ICP0) [2, 3, 22].

While comparative DNA sequencing of HVP2nv and HVP2ap isolates has revealed no significant subtype-specific amino acid differences in the US11, the amino acid sequences of the *vhs* and especially the ICP0 proteins diverge between HVP2 subtypes (unpublished data). Investigations are currently underway to determine if these two proteins play a role in the dissimilar replication of HVP2 subtypes in mouse cells.

In conclusion, all evidence to date points to the ability of an HVP2 isolate to grow in PMDF cells being an indicator of its ability to produce a rapidly lethal CNS infection in mice. This will allow *in vitro* refinement of experimental work prior to *in vivo* testing so that the number of mice needed for experiments can be greatly reduced. In addition, the PBDF and PMDF cell culture systems will be useful in discriminating between general defects in viral replication and species-specific restriction of viral replication, i.e., determining what viral-host interactions are involved in determining the outcome of cross-species infections. The primary dermal fibroblast cultures provide an excellent *in vitro* system with which to study aspects of the early, local, virus-host interactions that occur at the site of infection. These factors may play a critical role in determining the eventual outcome of the infection within the host.

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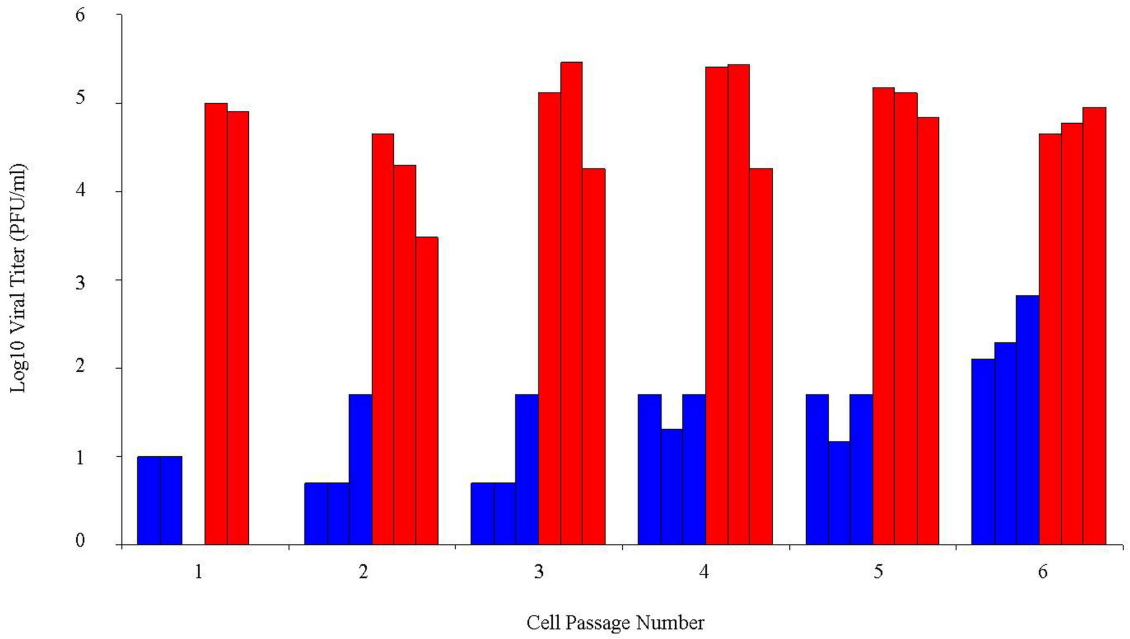

Figure 6.1 - Passage stability of PMDF cultures for virus replication. At each passage indicated, PMDF cultures were split 1:3 and infected with three strains of HVP2ap (■) or HVP2nv (■) at an MOI of 0.4 PFU/cell. At 3 days PI, cells were harvested in the media, freeze-thawed, centrifuged, and infectious virus present in the supernatant titrated by plaque assay.

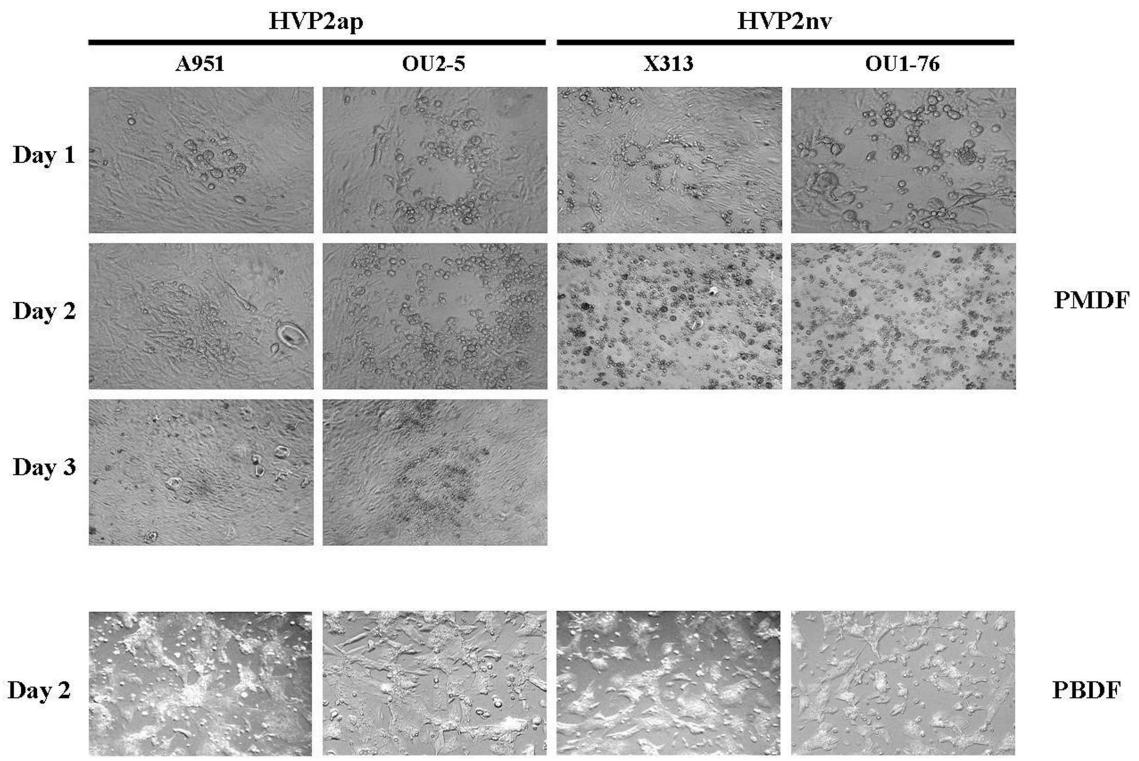

Figure 6.2 - Differential replication of HVP2 subtypes in PMDF vs. PBDF cell cultures. Confluent PMDF or PBDF monolayers were inoculated with virus at an MOI of 0.04 PFU/cell with two HVP2ap and two HVP2nv strains and photographed every 24 hours.

PMDF cultures - By 1 day PI both viral subtypes formed plaques although HVP2ap plaques were smaller compared to HVP2nv. By 2 days PI, HVP2nv strains completely destroyed the PMDF cell monolayers while the plaque size of HVP2ap plaques had decreased. While rounded up cells were still evident at 3 days PI in HVP2ap-infected PMDF cultures, all plaques filled in with uninfected cells.

PBDF cultures - all HVP2 isolates replicated with equal efficiency, producing 4+ CPE by 2 days PI.

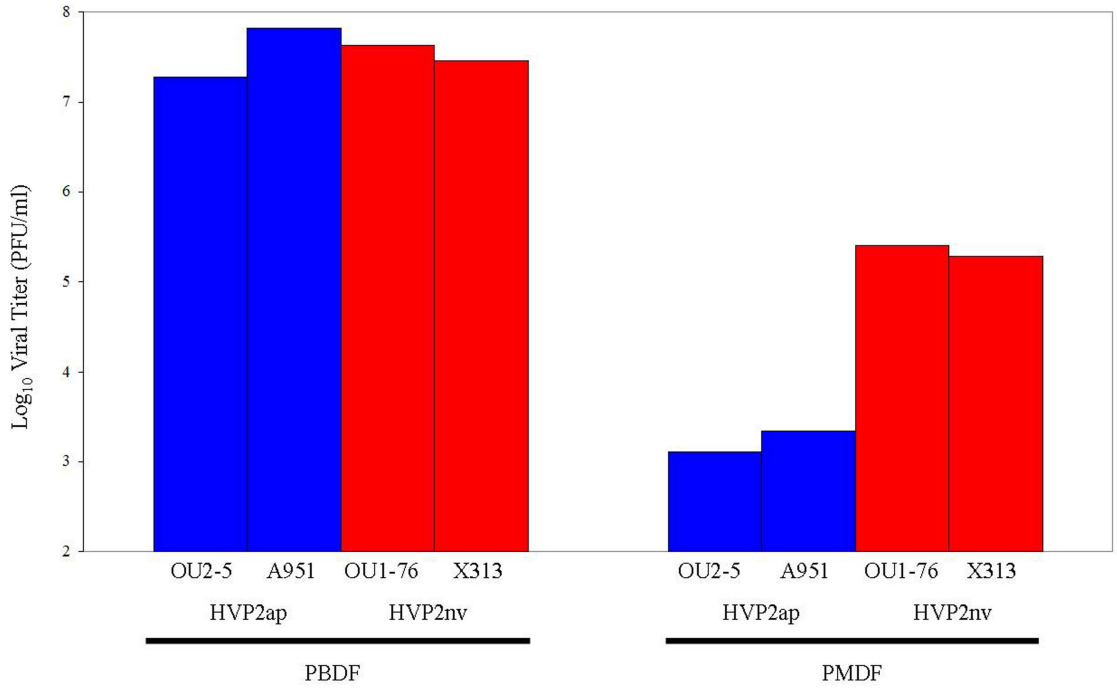

Figure 6.3 - Replication of HVP2 subtypes in PMDF vs. PBDF cell cultures. Cell cultures were infected with two strains each of HVP2ap (■) or HVP2nv (■) at an MOI of 0.04 PFU/cell. At 48 hours PI, cells were scraped into the media, freeze-thawed, centrifuged and infectious virus in the supernatant titrated by plaque assay. All viruses replicated equally well in PBDF cultures, but HVP2nv strains replicated to higher titers than HVP2ap strains in PMDF cultures.

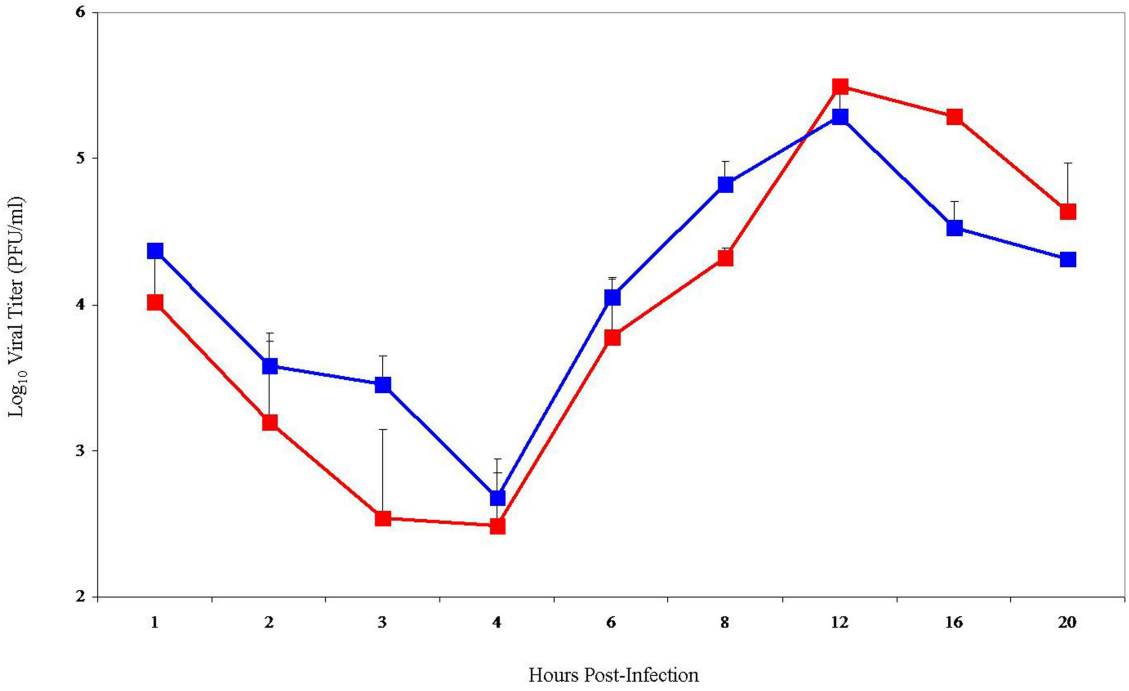

Figure 6.4 - One-step growth curve for HVP2 subtypes in PMDF cell cultures. PMDF cell monolayers were infected with 20 PFU/cell of two HVP2nv isolates or two HVP2ap isolates and incubated at 39 °C for 20 hours. Data points represent mean PFU values of two isolates from HVP2nv and HVP2ap at every time point for intracellular virus. There was no difference in the amount of infectious virus produced by HVP2nv vs. HVP2ap.

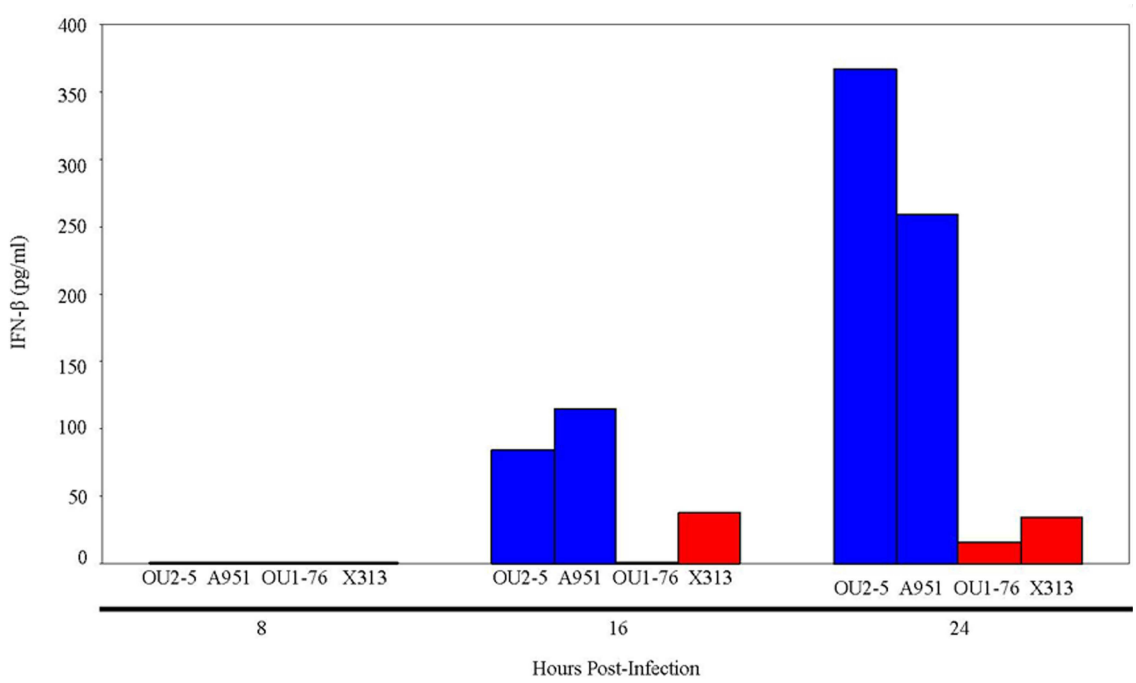

Figure 6.5 - IFN-β production in HVP2ap- vs. HVP2nv-infected cell cultures. PMDF cells were infected with HVP2ap strains OU2-5 and A951 (■) or HVP2nv strains OU1-76 and X313 (■) at an MOI of 1.2 PFU/cell. Media was collected at designated time points and the concentration of IFN-β in the extracellular media was quantitated by ELISA. No IFN-β was detected in uninfected PMDF cell cultures at any time point (data not shown).

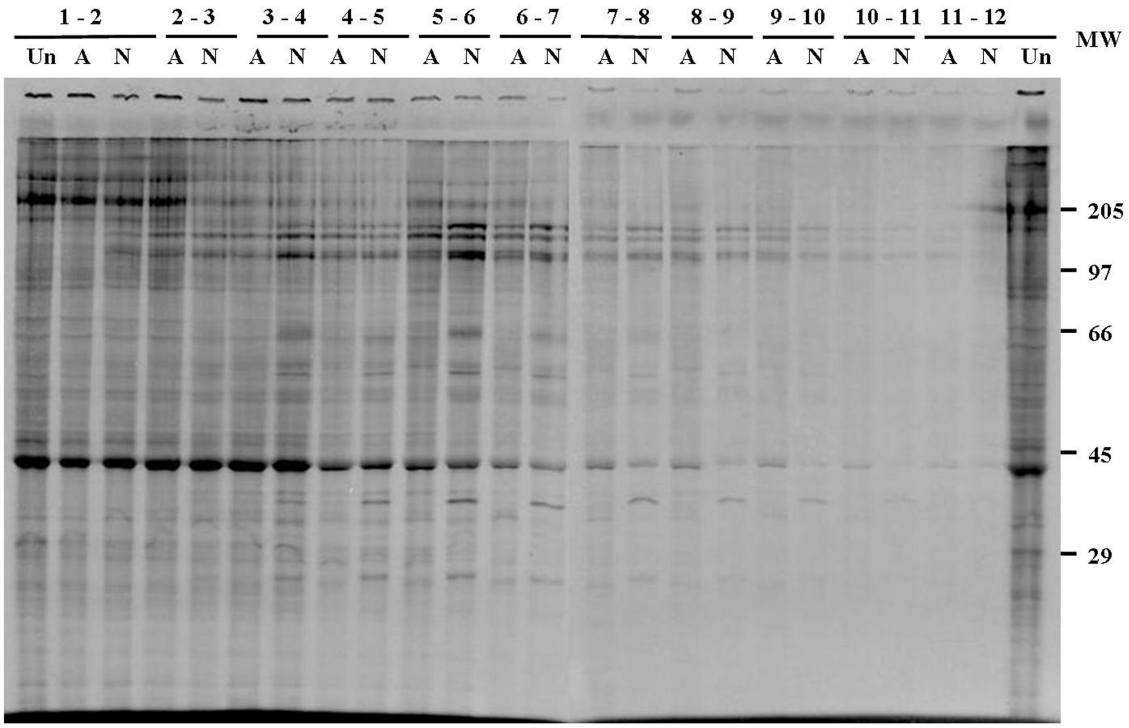

Figure 6.6 - Autoradiograph of protein synthesis in HVP2-infected PMDF cells. Confluent PMDF cultures were infected with HVP2ap strain OU2-5 (A) or HVP2nv strain OU1-76 (N) at an MOI of 4.0 PFU/cell. Cell cultures were labeled with ^{35}S-Met/Cys (30 µCi/ml) for 1 hour intervals as indicated and harvested. Uninfected cells (Un) were also labeled for reference. Proteins were separated by SDS-PAGE on a 10% gel. The position of molecular weight standards is shown at right.

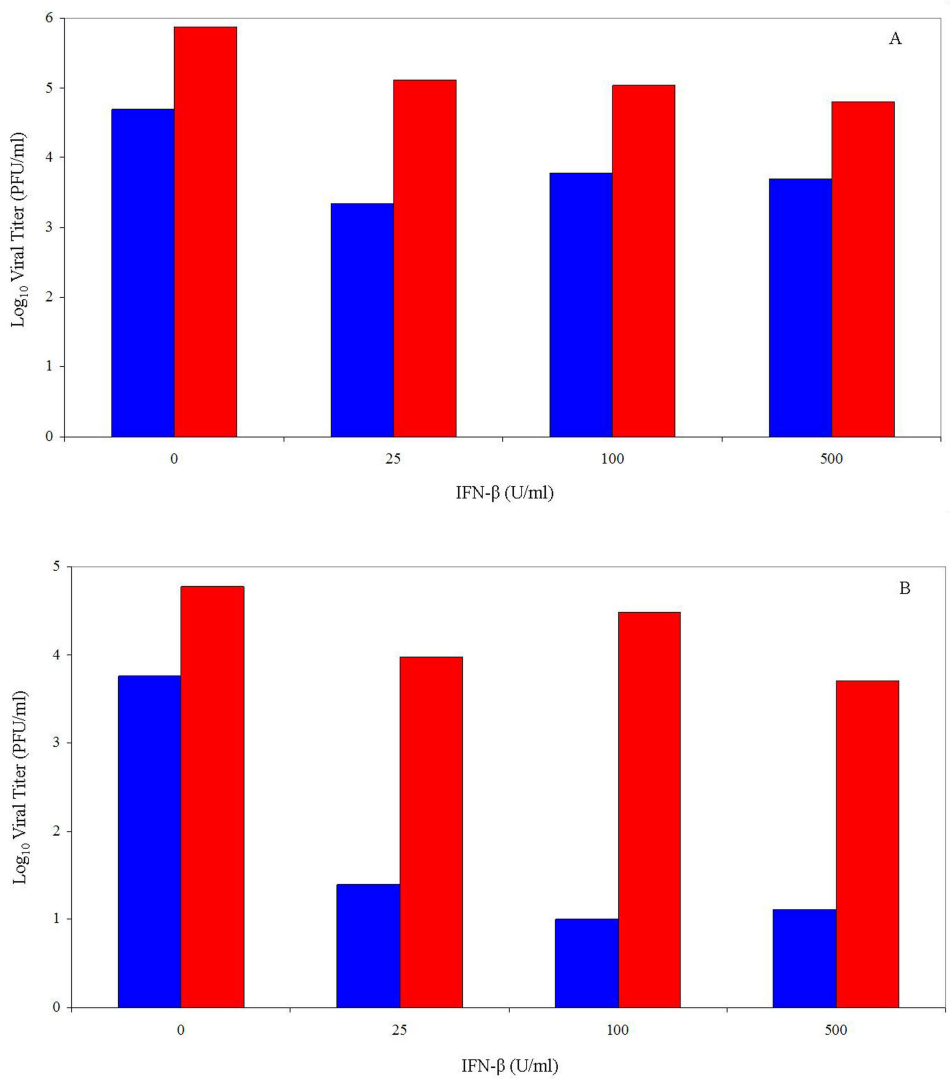

Figure 6.7 - Pretreatment of PMDF cells with IFN-β. PMDF cell cultures were pretreated with recombinant murine IFN-β at the concentrations indicated. After 18 h, cells were infected with HVP2nv strain OU1-76 (■) or HVP2ap strain OU2-5 (■) at an MOI of 4.0 (A) or 0.04 (B) PFU/cell. Infected cells were harvested at 24 hours PI and infectious virus titrated on Vero cells. Pre-treatment did not affect the production of infectious virus for either HVP2 subtype at an MOI of 4.0 PFU/cell. At the lower MOI while HVP2nv replication remained unaffected, HVP2ap replication was inhibited by the addition of exogenous IFN-β.

CHAPTER VII

SUMMARY AND CONCLUSIONS

The goal of this research was to identify sequences in the genome of *Herpesvirus papio* 2 (HVP2) that determine the outcome of cross-species infections in mice. Illumination of viral genetic elements which allow two closely related subtypes of a single virus to produce either extremely lethal CNS infections or infections that are clinically apathogenic will provide important information for vaccine design and treatment for other closely related, medically relevant, alpha-herpesviruses.

The first phase of experiments utilized DNA sequencing to compare specific genes of HVP2nv and HVP2ap. In general, most genes showed very little to no sequence variation that correlated with the pathogenic subtypes of HVP2 observed in mice. No readily identifiable, major sequence differences such as premature stop codons, frameshifts, or large insertions/deletions were observed in any HVP2 genes sequenced and analyzed.

Faced with the lack of obvious genetic differences in the genes examined, it became clear that further information was needed regarding the biology of the two HVP2 subtypes in both the natural host and cross-species infections. Studies were designed to provide an in-depth look at the *in vivo* behavior of these viruses over the course of an infection rather than just the endpoint value of death or survival. Information obtained

during the course of these experiments significantly narrowed the list of viral genes potentially responsible for the dichotomous pathogenicity of HVP2 subtypes in mice.

Initially it was important to determine if the HVP2 subtypes behaved differently during primary infection of their natural baboon host. Differences in the natural host would suggest a general replication deficiency for the HVP2ap isolates rather than a phenotype specific to cross-species infections. The experimental HVP2 baboon infections demonstrated that within the natural baboon host the two HVP2 subtypes are indistinguishable with regards to lesion development/severity, seroconversion, and shedding of infectious virus during primary infection.

An *in vivo* correlation to these baboon experiments was provided by infection of PBDF cell cultures prepared from the abdominal skin of a stillborn baboon infant with isolates of either HVP2nv or HVP2ap. By 48 hours PI, all isolates of HVP2 had replicated with equal efficiency and produced 4+ CPE in PBDF cell cultures. Titers of infectious HVP2 produced in PBDF cell cultures were identical for HVP2ap and HVP2nv isolates. Thus, there were neither quantitative nor qualitative differences observed between HVP2 isolates in a PBDF cell culture model system.

Results from the baboon and PBDF experiments provided evidence that no differences exist between HVP2 isolates within the context of the natural baboon host, either *in vivo* or *in vitro*. The initial characterization of HVP2 isolates as well as the baboon experiments detailed in this work demonstrated that both HVP2 subtypes are infectious in the natural baboon host (2, 5). Thus, whatever viral genetic elements determine the dichotomous pathogenic phenotypes of HVP2 isolates in mice appeared to

be specific for cross-species infections. The similar behavior of both HVP2 subtypes in baboons argues against a general deficit which decreases the ability and/or efficiency of HVP2ap isolates to replicate. In light of this information, HVP2 genes involved in basic viral replication are unlikely to be determinants of the differential pathogenicity of HVP2 subtypes in mice. Therefore, attention turned towards genes which play a more subtle role in enhancing the infectivity and virulence of herpesviruses. To further narrow the list of possible candidate genes, additional experiments were performed utilizing a mouse model system of cross-species HVP2 infection.

Initial experiments with HVP2 in mice used intra-muscular (i.m.) inoculation as the route of infection. The next step was to assess whether the pathogenic phenotypes observed after i.m. inoculation of mice remained consistent using additional routes of infection. Skin scarification (s.s.) inoculation proved to be an efficient and consistent route of HVP2nv infection in mice. However, similar to i.m. inoculation, HVP2ap was unable to produce CNS disease in mice following s.s. Results of the eye splash infection and intra-cranial (i.c.) inoculation both supported the existence of two pathogenic phenotypes of HVP2 in mice. Overall, it appears that once HVP2nv is able to gain access to the CNS, it results in a fulminant, rapidly fatal, encephalomyelitis in mice regardless of the route of entry (ie. epidermis, eye, muscle). The corresponding apathogenicity of the second HVP2 subtype in mice regardless of the route of inoculation suggested two possibilities: 1) HVP2ap is unable to replicate to sufficient titers at the initial site of inoculation for entry into the nervous system, or 2) HVP2ap is unable to replicate within tissues of the mouse nervous system. Subsequent experiments were designed to determine which situation existed in the mouse model system.

In order for HSV to initiate a CNS infection following peripheral inoculation the virus must overcome a threshold of replication at the epithelial site of infection (4, 6). Once in the PNS, virus enters afferent axons of sensory neurons for transport to neuronal nuclei in the ganglia. Within an infected non-natural host, continued viral replication in the sensory neurons can occur rather than cessation of viral gene expression and subsequent latency. Virus then travels back to the epithelium via sensory nerve axons, similar to what may occur during infection in a natural host. In a non-natural host, the virus may also travel via efferent axons into the CNS where continued replication allows spread throughout the spinal cord and brain. Viral replication within the CNS produces a severe inflammatory response which often contributes to the clinical signs of CNS disease and death. To determine which of these stages of infection differed between HVP2nv vs. HVP2ap, the spatial and temporal distribution of HVP2 infection in the mouse was characterized. Following s.s. inoculation with 10^5 PFU of HVP2nv or HVP2ap, apathogenic isolates of HVP2 replicated less efficiently at the site of inoculation and exhibited both a temporal delay of entry into the nervous system and significantly reduced titers within the CNS compared to HVP2nv. The temporal delay for CNS entry exhibited by HVP2ap compared to HVP2nv suggests that less efficient replication of HVP2ap at the site of inoculation requires more rounds of replication for it to reach the necessary threshold titer required for entry into the CNS. Once in the CNS, HVP2ap again replicated inefficiently, producing negligible quantities of viral DNA and only mild inflammatory lesions. Therefore, although both quantitatively and temporally deficient compared to HVP2nv, isolates of HVP2ap are neuroinvasive but appear to lack the capacity for neurovirulence.

To ascertain whether HVP2ap avirulence could be overcome by direct CNS inoculation, HVP2 was introduced directly into the brains of mice using intra-cranial (i.c.) injection. While all mice that received doses of HVP2nv as low as 10^2 PFU rapidly succumbed to infection, only 4/5 mice that received 10^6 PFU of HVP2ap died or showed signs of CNS infection. The time of death in these mice was very rapid; suggesting that the dose of virus was sufficiently high such that sustained viral replication in the CNS was not necessary to cause destruction of brain tissue and death. HVP2-specific immunohistochemical staining of brain tissue sections demonstrated appreciable quantities of viral antigen with widespread distribution throughout the brains of HVP2nv-infected mice. In contrast, only a minimal amount of antigen confined to the cerebral site of inoculation was detected in HVP2ap-infected mice which survived infection. The absence of virus-positive neurons or glial cells in the brains of HVP2ap-infected mice corroborated the inability of HVP2ap isolates to replicate and spread efficiently within brains of infected mice. These results taken together with evidence from the time-course study indisputably defined the pathogenic phenotype of HVP2ap in mice as neuroinvasive but non-neurovirulent.

At this point although valuable information had been obtained which characterized the *in vivo* behavior of HVP2ap in mice, the list of possible genes which could account for differences between HVP2nv and HVP2ap in mice remained daunting. Focus on construction and analysis of HVP2 subtype-specific recombinants was determined to be the most efficient means for determining which viral genes contributed to the dissimilar phenotypes of HVP2 subtypes in mice.

One foreseeable roadblock in analyzing HVP2 subtype-specific recombinants was the lack of an *in vitro* model system capable of differentiating between the two HVP2 subtypes. Without such a model, recombinant testing would be time consuming, expensive, and require large numbers of mice. Prior attempts to develop an *in vitro* model system for HVP2 infection using multiple continuous primate and mouse cells lines as well as commercially available mouse embryo fibroblasts had failed.

The preparation of PMDF cell cultures provided an ideal model system for differentiating between HVP2 subtypes *in vitro*. This model system will prove invaluable for the selection and analysis of HVP2-subtype recombinants which are currently being constructed. Further, accurate reflection of the differential pathogenicity of HVP2 subtypes in mice within this closed *in vitro* system provided valuable information about early, local, host-virus interactions that appear to be important determinants of the outcome of HVP2 infections in mice. First, critical host-virus interactions which may determine efficiency of HVP2 subtype replication at the epidermal site of inoculation in mice occur very early after infection. Analysis of protein synthesis within HVP2-infected PMDF cell cultures revealed that even at a high MOI, subtle differences are evident between HVP2 subtypes by 1-2 hours PI. This information seems to contradict the results from one-step growth curves which showed no difference in viral titer between HVP2ap- vs. HVP2nv-infected PMDF cells. However, slight differences in the efficiency with which the two HVP2 subtypes shut down host protein synthesis and produce viral proteins may be cumulative so that multiple rounds of replication are required to see the phenotype of decreased viral titers. Secondly, some host factors that contribute to the outcome of HVP2 infection at the site of inoculation are

present and functional within PMDF cultures. However, other host-specific factors as immune cells and soluble mediators which are recruited to the site of inoculation *in vivo* are absent in PMDF cultures. Thus, the inability of HVP2ap to replicate efficiently at the site of infection *in vivo* is likely due to factors that are present at the site of inoculation during early stages of HVP2 infection.

Within the context of the PMDF/HVP2 infection model, HVP2nv was shown to interfere with IFN-β production and to be less sensitive to the anti-viral effects of this important cytokine. The inability of HVP2ap to either prevent the expression of IFN-β and/or efficiently overcome the protective role of IFN-β may be one factor which explains why HVP2ap replicates and spreads poorly compared to HVP2nv following low MOI infection in PMDF cultures. High MOI infection infects a high percentage of cells in the initial round of infection, effectively eliminating uninfected bystander cells from the equation. At a lower MOI, substantially fewer cells are infected by the inoculum virus so that the ratio of uninfected to infected cells is high, and these uninfected cells become primed to resist infection through the action of IFN-β produced by infected cells. As HVP2nv reduces the amount of IFN-β produced and is able to productively infect IFN-β primed cells, the second phase of the IFN response in which IFN-β and IFN-α are both upregulated, is never initiated. The net result is that HVP2nv is able to replicate and spread unchecked in PMDF cell cultures. In contrast, INF-β is produced by HVP2ap-infected cells and appears to efficiently control viral replication and spread. Detection of increased levels of IFN-β between 16 and 24 hours PI suggests that the second wave of the IFN response does occur within HVP2ap-infected PMDF cell cultures. The "healing over" of HVP2ap plaques between 24 and 72 hours PI coupled with increased IFN-β

production and the sensitivity of HVP2ap to IFN-β all support the idea that HVP2ap is unable to overcome the host IFN-β response.

The inability of HVP2ap to sustain replication in PMDF cell cultures correlates with the lack of efficient, sustained, replication of HVP2ap *in vivo*. Experiments detailed in this work provide ample evidence for impaired replication of HVP2ap in mice regardless of the route of inoculation: negligible levels of viral antigen within infected tissues, mild to no lesions visualized at the epidermal site of inoculation, the inability to stimulate a strong, anti-HVP2-specific immune response, and the minimal number of viral genome copies detected within infected tissues.

HVP2/PMDF experiments were extended to include PMDF cells derived from different strains of mice including parental A129 and knockout 129 mice which do not express the IFN-α/β receptor ($IFNAR^{-/-}$). While *in vitro* studies of HVP2 infection utilizing the wild-type and knockout 129 PMDF cell cultures are in the very early stages, preliminary data on HVP2 replication in $IFNAR^{-/-}$ PMDF cells has been obtained. However, while *in vivo* HVP2 infections of A129 mice are indistinguishable from those seen in BALB/c mice (data not shown), assessment of HVP2 replication in A129 PMDF cell cultures is not completed. This data will be necessary to make the true comparison between the wild-type and knockout 129 cell lines.

Experiments with $IFNAR^{-/-}$ PMDF cell cultures were done to assess whether INF-β alone is sufficient to determine the outcome of HVP2 infection *in vitro* (Appendix I). Results suggest that IFN-β plays an important role in infection but that it alone does not determine the inefficient replication of HVP2ap *in vivo*. When analyzing these results, it

is crucial to remember that the PMDF cell cultures provide an isolated system that at best reflects only what occurs within cells at the site of inoculation; thus, PMDF cells are only a model for predicting the biology of HVP2 and the mouse host at the site of inoculation during the very early stages of infection. The fact that HVP2ap replicates much more efficiently in the knockout PMDF cells compared to BALB/c PMDF cells suggests that the IFN-β response is a crucial first line of defense against cross-species HVP2ap infection. Parallel *in vivo* experiments wherein the end result of infection depends on the full cascade of responses triggered by HVP2 infection and initiated by IFN-β may reveal that the IFN-β response is sufficient to prevent efficient replication of HVP2ap in mice. A cohort of IFNAR$^{-/-}$ mice is currently being produced to test this hypothesis *in vivo*.

Initially, microarray experiments were designed to analyze the mouse host gene expression profile in response to HVP2 infection. The goal of the project was to identify mouse genes which are differentially affected by HVP2nv vs. HVP2ap infection. Unfortunately, it was discovered that the mRNA:total RNA ratio is extremely low in primary skin cells. While infection of confluent PMDF cell cultures in 100 mm^2 plates resulted in high yields of clean, high quality total RNA, the quantity of mRNA in the samples was found to be too low for analysis. Further, 1000-fold amplification of the mRNA in each sample using a commercially available kit failed to increase the level of mRNA sufficiently to allow for microarray analysis. The low mRNA level was determined to be a property inherent to the cell types present in PMDF cultures and not caused by the virus infection as even uninfected cell mRNA did not reach the minimum threshold mRNA level for microarray analysis. Keratinocytes are one cell type present in the PMDF cultures which may have contributed to the low mRNA levels as they

constitutively display a low mRNA:total RNA ratio. Further, keratinocytes as well as other epidermal cell types produce numerous nucleases as a non-specific host defense mechanism. Resident RNase enzymes which were not efficiently inactivated during the RNA extraction process may have also contributed to low mRNA levels.

At the conclusion of this research project several viral genes have emerged as likely candidates to explain differences between HVP2ap and HVP2nv infections in mice. One possibility is the UL41 gene which produces the virion host shutoff (*vhs*) protein. When the virus first infects a cell and the virion envelope fuses with the cell plasma membrane, *vhs* is released from the viral tegument layer into the cytoplasm of the infected cell where it is responsible for cleaving both viral and cellular mRNA. The *vhs* protein is important for allowing the virus to take over host cell protein synthesis machinery for the production of viral proteins. This takeover results in the shut-down of host cell protein synthesis and thus reduces expression of important host cell mediators of innate immunity including IFN-β. Several lines of evidence resulting from studies with HSV support the role of *vhs* as a determinant of viral replication efficiency and pathogenesis: 1) HSV-1 and -2 UL41 mutants grow to significantly lower titers in mice with a corresponding decrease in their ability to induce disease; 2) UL41 mutants exhibit an MOI dependent phenotype *in vitro*, 3) replication/virulence of mutants is restored in $IFNAR^{-/-}$ mice, $IFNAR^{-/-}$ embryo fibroblast cultures, or following antibody neutralization of IFNα/β, and 4) HSV-2 ΔUL41 mutants produce more IFN-β and are more sensitive to IFN-β pretreatment than wild type virus (1).

While DNA sequencing did not reveal any significant subtype-specific differences and northern blot analysis of HVP2-infected PMDF cells showed no difference between HVP2 subtypes in the degredation of cellular β-actin mRNA at 4 hours PI, the phenotype of HSV UL41 mutants parallels what is observed with HVP2ap both *in vitro* and in mice. Further, *vhs* is expressed late in infection for packaging into nascent virions. Interaction between the newly synthesized *vhs* and the viral transactivator VP16 prevents *vhs*-mediated degradation of late viral transcripts. Two non-conservative amino acid changes (LAALDPP → PAALDPL) were identified just outside of the VP16 binding domain of *vhs*. These changes were found in all HVP2nv isolates sequenced but only 2/3 HVP2ap isolates such that they could not be categorized as subtype-specific. However, if these changes altered secondary structure and somehow decreased the VP16-*vhs* interaction, it could have deleterious consequences for the apathogenic isolates or somehow enhance replication in HVP2nv strains.

At this time, an HVP2nvΔUL41 virus has been produced as outlined in Appendix II. Northern blot analysis revealed no decrease in β-actin mRNA for HVP2nvΔUL41-infected PMDF cell cultures compared to uninfected PMDF cells. Infections of BALB/c PMDF cell cultures revealed that HVP2nvΔUL41 does not replicate as efficiently as either HVP2nv or HVP2ap such that the the replicative efficiency of HVP2ap is intermediate to HVP2nv and HVP2nvΔUL41. Similar to HVP2ap at and MOI of 0.01 PFU/cell, HVP2nvΔUL41 formed plaques by 24 hours PI but these plaques ceased to expand and subsequently filled with new cells to restore the monolayer between 48-72 hours PI. HVP2nvΔUL41-infected PMDF cells produce levels of IFN-β comparable to levels detected in HVP2ap-infected PMDF cultures.

In IFNAR$^{-/-}$ PMDF cell cultures, HVP2nvΔUL41 replicates to higher titers than in BALB/c PMDF cell cultures; however, again titers of HVP2nvΔUL41 are less than those observed for either HVP2 subtype. Therefore, it appears that deletion of UL41 from HVP2nv produces a virus which replicates *in vitro* in a manner similar to HVP2ap isolates. DNA sequencing characterized an intact ORF for UL41 in both HVP2 subtypes and no subtype-specific changes were identified in the 5' or 3' non-translated regions of UL41. Thus, the proposed phenotype for HVP2ap is one of a mutant rather than a knockout virus. The decreased replication efficiency of HVP2nvΔUL41 compared to HVP2ap in PMDF cell cultures is what would be predicted if mutation rather than deletion of HVP2ap UL41 were responsible for dictating the efficiency of HVP2ap replication. Currently, the HVP2 UL41 subtype recombinant (HVP2nvUL41ap) and HVP2nv revertant are being constructed and analyzed.

A second possible candidate is the diploid RL2 gene which produces the viral ICP0 protein. The HSV-1 ICP0 inhibits induction of interferon stimulated gene expression that is triggered by viral entry and is responsible regulating steady-state levels of cellular and viral proteins. HSV-1 ICP0 null mutants grow poorly *in vitro* at low MOI, a phenotype which is readily apparent in primary cell cultures and much less severe in continuous cell types (7). Further, HSV-1 ICP0 is essential for viral resistance to IFN-α/β *in vitro* and it has been suggested that the rate of ICP0 expression *in vivo* may be critical in determining whether host IFN can decrease viral titers and prevent disease (3).

Although DNA sequencing of the RL2 coding sequence revealed a great deal of variance between individual isolates, all isolates had the coding sequence to produce full-

length, properly spliced, ICP0 proteins. Again, this would suggest that if RL2 function or efficiency is altered between HVP2 subtypes the differences result from a more subtle mutation rather than lack of an intact RL2 ORF. While there were no subtype-specific amino acid differences in the RL2 coding sequence, the high sequence variability between individual HVP2 isolates may be masking changes in HVP2ap which alter characteristics important to the function of the HVP2ap ICP0 protein. An HVP2nvΔRL2 virus has been constructed as detailed in Appendix II and the virus is currently being plaque purified for use in experiments. Production of this null mutant was made more difficult by the diploid nature of the gene in the HVP2 genome. Southern blot analyses will be critical to ensure that both copies of the RL2 have been replaced by the GFP coding sequence.

DNA sequencing coupled with the series of *in vitro* and *in vivo* studies demonstrate that HVP2ap replicates less efficiently in primary mouse cells than does HVP2nv, regardless of the route of inoculation. *In vivo* and *in vitro* baboon/HVP2 experiments confirm that within the natural host, HVP2 subtypes are indistinguishable. Neurovirulent strains of HVP2 exhibit a pathogenesis in mice which parallels that observed in human BV infections. Thus, the HVP2nv/mouse model provides a safe, informative, and reliable model system for studying the complex interplay between virus and host that ultimately determine the outcome of cross-species and zoonotic infection.

The virulence of any given virus within a particular host is determined by the complex relationship that exists between the two entities. Alpha-herpesviruses are successful pathogens because they have co-evolved with their natural host to achieve a

state of balance which ensures survival of both the natural host and the virus. Any number of genetic differences may arise within individual viral strains which do not alter the behavior of the virus in its natural host, yet inadvertently increase/decrease virulence of the virus in a non-natural host species. These pathogenic differences can be attributed to the array of host-virus interactions which differ substantially between the infected natural vs. non-natural host species. Although the rate of mutation for dsDNA viruses is typically thought to be very low, subtle mutations which do not affect the virus within the context of its natural host are likely to be quite common. These changes may persist unnoticed until virus is examined within the context of a non-natural host species. Therefore, HVP2ap may actually represent a collection of mutant viruses with numerous possible genetic lesions, all of which independently result in a similar phenotype of avirulence in mice. However, from the standpoint of determining genetic mechanisms that enable viruses such as BV to produce severe, often lethal CNS infections when transmitted to a non-macaque host, these viruses are worthy of further investigation.

In conclusion, while no single HVP2 gene has been identified as being responsible for determining the dichotomous pathogenicity of HVP2 subtypes in mice, invaluable information about the biology of these viruses has been revealed throughout the course of these experiments. With the fundamental research complete, construction of HVP2 subtype recombinants will be an efficient and informative manner to determine which viral genetic elements contribute to the disparate pathogenicity of HVP2 subtypes observed in cross-species infections of mice.

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APPENDIX I

Preliminary Results for HVP2 Infection of IFNAR$^{-/-}$ PMDF Cells

To assess whether IFN-β alone was sufficient to determine the outcome of *in vitro* HVP2 infection, PMDF cells were prepared from knockout mice. Like the mice, these cells do not express the IFN-α/β receptor (IFNAR$^{-/-}$). IFNAR$^{-/-}$ mice were generously provided by Dr. H.W. Virgin (Washington University School of Medicine, St. Louis, MO).

As shown in Figure A1.1, HVP2ap replicated to significantly higher titers in IFNAR$^{-/-}$ PMDF cells than in BALB/c PMDF cells at all MOIs tested. While the amount of infectious HVP2ap produced in BALB/c PMDF cells at an MOI of 1 PFU/cell was over 10-fold less than infectious HVP2nv, infection of IFNAR$^{-/-}$ PMDF cell cultures produced near equivalent titers of HVP2ap and HVP2nv. However, at lower MOIs HVP2ap were still unable to replicate as well as HVP2nv. Therefore, while IFN-β appears to play a role in the disparate outcome of *in vitro* infection with HVP2 subtypes, additional as yet unknown factors contribute to the ability of HVP2nv to replicate to higher titers compared to HVP2ap.

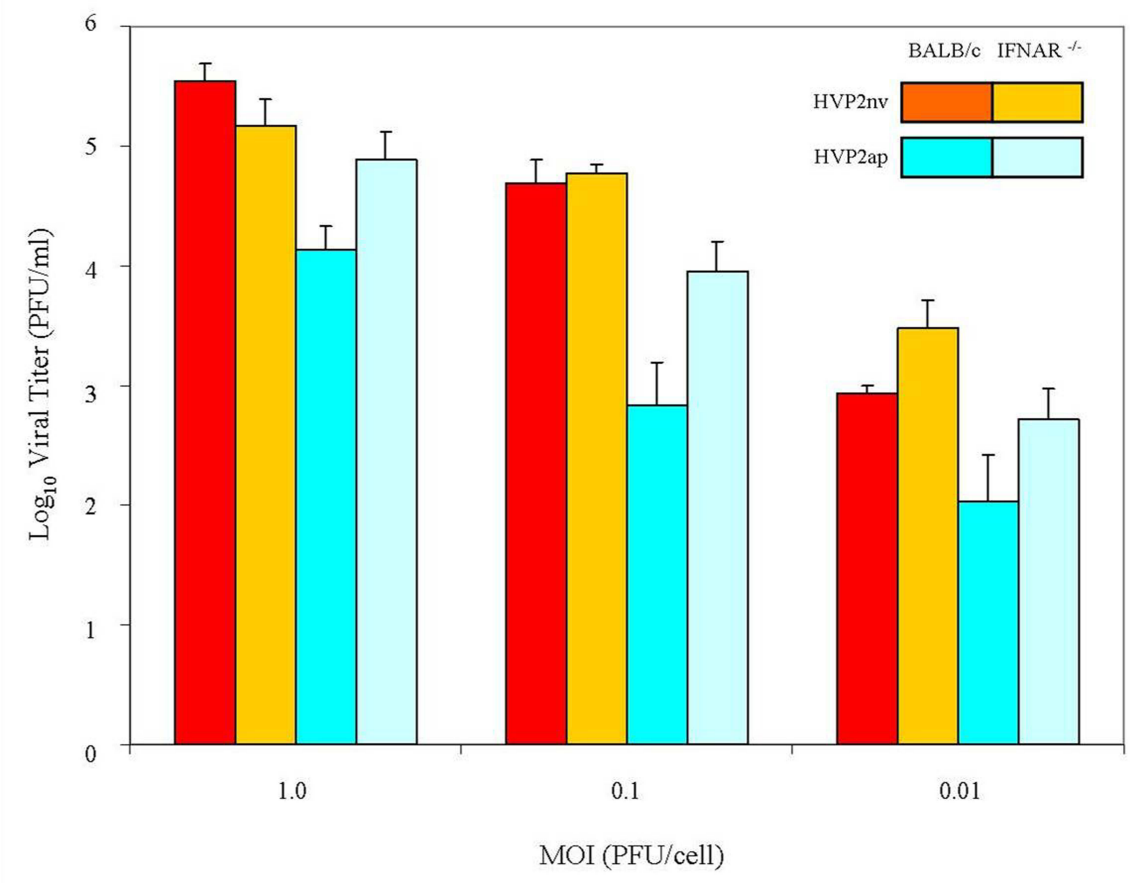

Figure A1.1 - Replication of HVP2 subtypes in BALB/c vs. IFNAR$^{-/-}$ PMDF cell cultures. Confluent BALB/c or IFNAR$^{-/-}$ PMDF cell monolayers were inoculated with HVP2nv isolate OU1-76 or HVP2ap isolate OU2-5 at an MOI of 1.0, 0.1, or 0.01 PFU/cell. At 24 hours PI, cells were scraped into the media, subjected to three rounds of freeze/thaw, centrifuged, and infectious virus in the supernatant titrated by standard plaque assay. Data points represent the average of replicate experiments (n=3) with standard deviations indicated by error bars.

APPENDIX II

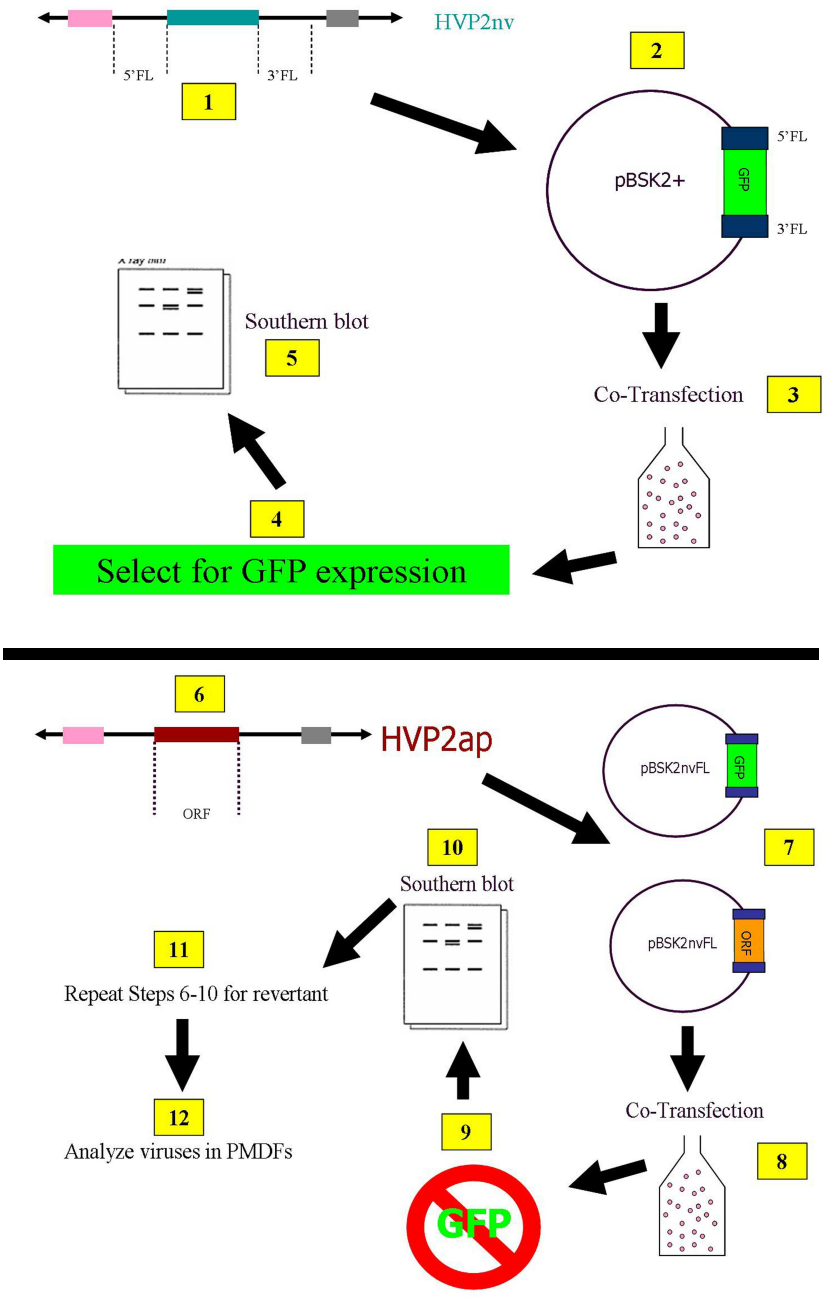

Figure A2.1 - Procedure for Constructing HVP2 Subtype Recombinants

1. PCR HVP2nv 5’ and 3’ flanking sequence from gene of interest engineering in appropriate restriction sites.
2. Digest PCR products with appropriate restriction enzymes and ligate into the pBlueScript2+ (pBSK) vector at the 5’ and 3’ termini of the GFP ORF. Sequence to confirm correct orientation and sequence of flanking sequences in pBSK vector (pBSK2nvFL).
3. Co-transfect 500 ng each of pBSKnvflank plasmid DNA and gradient purified HVP2nv DNA into 50-60% confluent Vero cells using the Gene Porter Transfection System (Gene Therapy Systems, Inc., San Diego, CA).
4. Harvest transfected cells when infection reaches 4+ CPE; plate dilutions of recombinant stock and plaque purify GFP+ plaques (HVP2nvΔGFP virus).
5. Prepare infected cell-DNA for Southern blot analysis to confirm the genomic location of the GFP ORF and the absence of target ORF in the knockout virus.
6. PCR the HVP2ap ORF for gene of interest incorporating the appropriate restriction sites.
7. Replace the GFP ORF in pBSK2nvFL with the HVP2ap ORF (pBSKapORF).
8. Co-transfect 500 ng each of the pBSKapORF plasmid DNA and gradient purified HVPnvΔGFP DNA.
9. Harvest transfected cells when infection reaches 4+ CPE; plate dilutions of recombinant stock and plaque purify non-florescent (GFP-) plaques.
10. Prepare infected cell-DNA for Southern blot analysis to confirm location of the target gene in the recombinant virus.
11. Repeat steps 6-10 to generate revertant virus except PCR HVP2nv ORF.

12. Test knockout, recombinant and revertant viruses in PMDF cell model system.

VITA

Kristin Michelle Rogers

Candidate for the Degree of

Doctor of Philosophy

Thesis: MECHANISMS RESPONSIBLE FOR THE DISPARATE PATHOGENICITY OF *HERPESVIRUS PAPIO* 2 SUBTYPES IN MICE

Major Field: Veterinary Biomedical Science, Molecular Virology

Biographical:

Personal Data: Born in Mesa, Arizona on January 6, 1970, the daughter of John and Roxanne Long. Married David Rogers on January 6, 2001.

Education: Graduated from Prescott High School, Prescott, Arizona in May 1988; received Bachelor of Science degree in Microbiology from Oklahoma State University, Stillwater, Oklahoma in May 2002. Completed the requirements for Doctor of Philosophy degree in Veterinary Biomedical Science at Oklahoma State University in December 2006.

Experience: Research Technician at Food and Agricultural Products Center, Oklahoma State University, Stillwater, Oklahoma, 2001. Research Assistant in Virology Laboratory of Dr. Alexander Lai, Microbiology Department, Oklahoma State University, Stillwater, Oklahoma, 2001. Graduate Research Assistant, Molecular Virology Laboratory of Dr. R. Eberle, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma, 2002-2006.

Professional Memberships: American Society of Virology, American Society for Microbiology.

Name: Kristin Michelle Rogers Date of Degree: December, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: MECHANISMS RESPONSIBLE FOR THE DISPARATE PATHOGENICITY OF *HERPESVIRUS PAPIO* 2 SUBTYPES IN MICE

Pages in Study: 245 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Science, Molecular Virology

Scope and Method of Study: The purpose of this study was to identify viral determinants of the neuropathogenesis or avirulence of *Herpesvirus papio* 2 subtypes (HVP2nv and HVP2ap) in BALB/c mice. Experiments were conducted to determine if HVP2 subtypes were similar within the natural baboon host. Focus then turned to characterizing the temporal and spatial distribution of HVP2 subtypes within the central nervous system (CNS) of mice following different routes of inoculation. Serum anti-HVP2 IgG levels were measured by ELISA and tissues were examined microscopically with routine stains and immunohistochemistry. HVP2-specific quantitative real-time PCR was used to determine the amount of viral DNA within infected mouse tissues at various times post-infection. Primary mouse dermal fibroblast (PMDF) cell cultures were developed as a model system to examine early host responses to viral infection at the site of inoculation.

Findings and Conclusions: HVP2 subtypes were indistinguishable in the natural baboon host. Experimental HVP2nv infection of mice produced a fulminant, fatal encephalomyelitis for all routes of inoculation tested: intra-muscular, intra-cerebral, eye splash, and epidermal scarification. Mice infected with HVP2ap in the same manner showed no clinical signs of CNS infection. Minimal HVP2ap replication at the site of inoculation and within the CNS was evidenced by mild, primarily immune-mediated lesions in which viral antigen and viral DNA were conspicuously absent. Only mice that received 10^6 PFU of HVP2ap developed CNS disease following intra-cerebral inoculation. Thus, HVP2 is neuroinvasive but not neurovirulent. Experiments in PMDF cultures revealed differential responses of HVP2 subtypes to IFN-β: HVP2nv decreases INF-β expression and evades its anti-viral effects while HVP2ap-infected PMDF cells produce appreciable quantities of IFN-β resulting in decreased titers. Differences in the host-virus interactions largely determine the outcome of HVP2 infection in mice.

ADVISER'S APPROVAL: R. Eberle