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INNATE AND ADAPTIVE HOST RESPONSE DURING THE INITIAL PHASE
OF HERPES SIMPLEX VIRUS ENCEPHALITIS IN THE NEONATAL MOUSE

To study early events of neonatal Herpes Simplex Virus (HSV) encephalitis and its sequelae we induced a controlled infection in the brains of mice using HSVgH-, a genetically modified Disabled Infective Single Cycle virus. Neonatal Balb/C mice were infected with various amounts of HSVgH- virus by intracerebral injection. Results showed that the survival of infected mice was dependent on the amount of virus injected. Infection with 200,000 plaque forming units (pfu) of HSVgH- virus resulted in 0% survival, whereas 25,000 pfu resulted in 75% survival. If the mice died, 98% of the deaths occurred between 3 and 7 days after infection. Replication competent virus was recovered from 20% of mice brains infected with 25,000 pfu of HSVgH-. Neutralizing antibodies were not detected 6 weeks post infection in sera of mice, which survived infection with 25,000 pfu of HSVgH-. LacZ histochemistry and immunoperoxidase staining using anti-HSV and anti-beta-galactosidase antibody revealed that the infection was limited to the site of injection. Tissue destruction was observed at the site of inoculation 3 days post infection using cresyl violet staining. At 3 days post infection adjacent sections showed positive cells for viral antigens and apoptotic cells in the infected area. Immunoperoxidase staining using antibodies to surface markers showed microglial activation beginning on day 1 and astrocyte proliferation beginning on day 3-post infection. B and T lymphocytes were not

detected on day 1 through 7-post infection. This controlled experimental HSV infection suggests a limited non-specific early host response of innate immunity and absence of adaptive immunity in the neonate to HSV encephalitis.

Innate and Adaptive Host Response During the Initial Phase of Herpes Simplex Virus
Encephalitis in the Neonatal Mouse

A dissertation submitted to Kent State University
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CHAPTER 1

Introduction

Herpes Simplex Virus:

Herpes Simplex virus (HSV) is a DNA virus belonging to the family of Herpesviridae (Wildy P 1973). There are two types of HSV aptly named type 1 (HSV-1) and type 2 (HSV-2) both structurally very similar but differ in genomic composition. The HSV virion consists of an envelope, tegument, capsid and a core containing the viral genome (Figure 1). The outer envelope is made up of lipid and is known to be derived from patches of altered cellular membranes, with a characteristic trilaminar shape. The envelope is studded with more than 1000 copies of at least 10 different types of protruding glycoproteins, most of which are essential for the viral infection and replication. The tegument, which is an amorphous material, is present inside the envelope between the inner layer of the envelope and the protein capsid. The distribution of tegument is asymmetrical and the thickness varies with the location, thus causing variation in the size of herpes virions. The protein capsid is icosadeltahedral in shape. It is 162 nm in diameter and it is made up of 162 capsomeres. The overall HSV virion is made up of 35-45 different polypeptides. Inside the capsid lies the core containing the viral DNA in form of a torus (Roizman B and Sears AE., 1996).

The herpes viral genome is a linear double stranded DNA measuring 152 kbp in length, basically consisting of a Long region (L) covalently linked to a Short region (S) (Figure 1). Each arm is composed of a unique sequence (U_L and U_S) flanked by long inverted repeat sequences. The HSV genome contains 84 known genes (Ward PL and Roizman B, 1994) of which 45 are essential for virus replication (Roizman B and Sears AE., 1996).

HSV-1 and HSV-2 both are known to cause gingivostomatitis, tonsillitis, labialis, pharyngitis, genital herpes and herpetic whitlow or HSV skin infection. Infections caused by HSV-1 alone include encephalitis, ketatoconjunctivitis, esophagitis, tracheobronchitis and herpes gladiatorum. Infections caused by HSV-2 alone include meningitis and perianal herpes. HSV-1 most commonly causes infections above the waist whereas HSV-2 most commonly causes infections below the waist. The infections are usually severe in immunocompromised as well as the neonates (Whitley RJ, 1996).

a

Reproduced from Dr.Edward Wegner's page

b

bb' and cc' : Terminal repeat sequences

U_L: Unique sequence of the long region

U_S: Unique sequence of the short region

Reproduced from Fields Virology (4th edition).

Figure 1 : Structure of Herpes Simplex Virus (HSV)

a. Structure of HSV Virion

b. Schematic illustration of HSV genome.

Neonatal HSV infections:

Epidemiology and clinical features:

Neonatal HSV infections occur at a rate of 1 in every 1500-3000 births in United States (Sullivan-Bolyai. J. et al., 1983). The majority of HSV infections in neonates are acquired during passage through the birth canal of a previously infected mother. However 20% of infections are acquired as a result of post-natal infections from non-maternal sources. Encephalitis can be a result of blood borne dissemination, which results in multifocal lesions. In contrast, neonates who present with only encephalitis develop the disease as a consequence of retrograde axonal transport. HSV infections in the neonate can cause three types of disease: 1. Encephalitis 2. Disseminated disease involving multiple organ system involvement; or 3. Skin, eye and mouth (SEM) disease (Whitley RJ, 2001). A great majority of the disseminated multiple organ system disease and SEM evolve into encephalitis. Therefore encephalitis is the most common outcome of neonatal HSV infections (Whitley RJ et al., 1980).

Neonatal encephalitis due to HSV is a devastating disease and if untreated results in >50% mortality and permanent neurologic sequelae in 75% of survivors. Treatment with intravenous acyclovir has reduced the mortality from >50% to 15%, though 50% of treated survivors still have neurologic impairment (Sullivan-Bolyai. J et al., 1983). As a result of improvement in antiviral therapy, a high proportion of neonates with HSV infections are surviving, but require long term follow up because of the neurologic

sequelae. In spite of early intervention there has been a failure to achieve return to normal, reflected by long-term morbidity (Whitley RJ, 1993; Whitley R et al., 1991).

Effects of HSV infections of CNS:

HSV infections of CNS result in extensive tissue damage due to hemorrhagic necrosis with a predominant lymphocytic infiltration (McDougal R.A. et al., 1954, Singer DB 1981). There are two contributory factors involved in the cellular destruction namely the replication of the virus resulting in cell death and the subsequent immune/inflammatory response. The viral replication initially induces apoptosis but at a later stage the virus produces many proteins which interfere with apoptosis (Goodkin ML et al., 2004). It is not entirely clear whether apoptosis or necrosis is the actual mechanism of cell death (Geiger KD 1997 et al.; DeBiasi RL et al., 2002; Shaw MMW et al., 2001)

HSV is capable of infecting most of the nucleated cells in the body. HSV infects neurons, astrocytes and oligodendrocytes in the CNS culminating in death of the infected cell. The infected cell undergoes various structural and biochemical changes as the cell macromolecular synthetic machinery is taken over for the purpose of viral replication. Some of the earliest changes are the disaggregation of the nucleolus, condensation and margination of host chromosomes and cytoskeletal destabilization. Viral glycoproteins are inserted in the plasma membrane of the infected cell. The DNA and protein synthesis

of the host cell is shut off and as the infection proceeds host mRNA is degraded (Aubert MJ et al., 1999).

HSV is also capable of non-productively infecting neurons and entering a stage of latency where the virus is quiescent, does no damage to the cell and the only evidence of its presence is the latency associated transcript (LAT)(Bloom DC, 2004). LAT is an abundant stable intron that accumulates in the nuclei of latently infected neurons of infected animals and humans. The function of LAT and its relationship to latency is yet to be determined. Latent HSV in the neuron can be reactivated under favorable conditions and cause lytic infection and cell destruction. In case of neonatal HSV infections, this pattern maybe followed but only if the child survives the initial productive infection.

Host response to HSV infection:

The CNS is an immunologically privileged site and the host response to cellular injury at this site differs from the response in other sites. First there is a blood brain barrier which when functioning normally prevents white blood cells and antibodies from entering the CNS. Neuronal cells express low levels of MHC I molecules to reduce T cell targeting (Rall GF, 1998), and CNS may have mechanisms to promote apoptosis of T cells that enter the brain (Bauer J et al., 1998) and promote the generation of a Th2 response instead of a Th1 response to the infection (Irani DN et al., 1997). But most of

these mechanisms are either not very well developed in neonatal brain or can be altered easily by adverse conditions such as herpes encephalitis.

The inflammatory response to CNS infection caused by HSV contributes to CNS destruction as well as the infection itself. Studies with HSV infection in adult animals have shown that after initial polymorphonuclear neutrophil reaction there is reactive astrogliosis, microglial activation and T-cell recruitment to the infected brains at a very early stage in the infection (McKie EA et al., 1998). Other studies with intracerebral infection of mice using mutant herpes simplex virus with limited pathogenesis have shown that T-cell and inflammatory responses induce the release of inflammatory cytokines which contribute to the tissue damage (Irani DN, 2001). But in case of neonatal herpes encephalitis there is limited information regarding the host response during the early stages in the disease. Moreover activation of neurohumoral response in neonatal brain in form of astrogliosis and microglial proliferation as well as its role in neuroprotection is not completely understood. But it is generally understood that the neonatal animals lack the capability to mount an adaptive immune response either in form of cell mediated or humoral response to HSV infection.

General problems in management of Neonatal Herpes encephalitis:

The approach in management of neonatal herpes encephalitis has improved significantly over the years. But the early diagnosis and long term management still continue to be a problem most commonly because the signs and symptoms may be scarce at the earlier stages. Most of the mothers of HSV infected newborns are asymptomatic (Whitley RJ et al., 1980). In HSV encephalitis it is usually the cerebral cortex and rarely the brainstem which is affected. Since the neonates do not require all the cortical functions for most of the activities, HSV replication within the cortex can be mostly asymptomatic (Arwin AM et al 2001, Whitley RJ et al., 1980, Whitley RJ 1983, Corey L et al., 1988, Whitley R et al., 1991). CNS infection of HSV can be difficult to diagnose by CSF examination, because the meninges are not affected during the initial stages of infection (Arvin AM et al., 2001; Whitley R et al., 1991; Whitley RJ et al., 1988).

Amplification of HSV DNA in CSF by PCR is the most sensitive method for diagnosis of HSV (Kimberlin DW et al., 1996; Aurelius E et al., 1991). But the sensitivity of this diagnostic procedure is only ~70% in early stages of infection (Kimberlin DW et al., 1996) compared to ~100% after the onset of symptoms (Aurelius E et al., 1991; Studahl M et al., 1998; Weil AA et al., 2002). These factors hinder the early diagnosis of neonatal herpes encephalitis at an early stage where therapeutic intervention could limit the long term damage.

Limitations of the study of HSV CNS infection:

Induction of limited CNS lesions by HSV infection, in an animal model is difficult because HSV is a neurotrophic virus which normally causes rapid destruction of the CNS and death. For the same reason, there are very few studies describing herpes encephalitis in a neonatal animal. An infection model described in owl monkey (*Aotus trivirgatus*) has served as the standard non-human primate model of herpes simplex virus-1 (HSV-1) infection because it is highly susceptible to HSV-1 encephalitis (Katzin, D.S. et al., 1967). Owl monkeys are expensive, difficult to obtain difficult to maintain in captivity, and not all the laboratories are equipped to maintain them. Another model was described which is the common marmosets where it was determined that they are equally susceptible to HSV infection. In both the described models, infection resulted in high mortality (Deisboeck, T.S. et al., 2003). Moreover both these animal models pose many practical problems and cannot be used in larger numbers for studying the disease.

Another study on the effect of HSV infection in neonatal rat brain on sensory gating describes a poorly characterized rodent model of neonatal herpes encephalitis. In this model the infection was induced using small amounts of virus which did not result in any detectable lesion. The survival studies determined that intracerebral infection with wild type HSV-1 virus in amounts of 2.5 plaque forming units (pfu) resulted in death of 60% of infected neonatal rats and infection using 0.6 pfu resulted in ~35% mortality (Engel JA et al., 2000).

Specific goals:

To study the initial events of neonatal herpes encephalitis the following goals were established:

1. To develop an animal model: The existing models were not practical to study the disease and therefore this study was designed to develop a mouse model of neonatal herpes encephalitis with the following requirements. The infection induced should:

- (i) be non-lethal to majority of infected mice.
- (ii) be limited and controlled; and
- (iii) cause detectable lesions.

2. Characterize the host response to the CNS lesions induced by HSV

- (i) Humoral response
- (ii) Neuroimmune response- Astrocyte and microglial response
- (ii) Adaptive immune response –T and B lymphocyte recruitment in the infected

brain

Hypothesis:

Previous studies suggested that the use of a wild type virus with uncontrolled replication capabilities could not be used to study early events in HSV induced encephalitis. Therefore a defective HSV was employed. There are many defective HSV strains used as vectors and in cancer therapy in animal models which cause non-lethal CNS infection (McKie et al, 1998) and an inflammatory response similar to wild type HSV infection (McKie et al., 1998, Nguyen et al., 1992, Wood MJ et al., 1994, Wood MJ et al., 1994). Since defective virus is more likely to cause a limited, non-lethal infection than a wild type virus, the hypothesis is that:

Intracerebral infection of neonatal mice using appropriate amounts of a replication defective Herpes Simplex Virus will result in a limited non-lethal infection with detectable lesions, enabling the study of the host response.

In order to test the hypothesis three different defective HSV were utilized. Although defective, two of the three viruses that were tested could not be used due to several practical problems with them and their high neurovirulence in neonatal mice. But one was used successfully eliminating these problems and was used to test the hypothesis. This virus, HSVgH-, allowed the formation of a detectable HSV infection without killing the animal, enabling us to study the host response.

HSVgH-:

HSVgH- is a replication defective HSV, lacking the essential surface glycoprotein gH which is vital for viral attachment to the target cell membrane. This virus is also called SC16ΔgH because a 1110 base pairs in the gH encoding gene in a SC16 strain of HSV-1 was replaced with a LacZ encoding gene downstream of a cytomegalovirus IE1 promoter (Fig. 2a). These viruses are cultivated in complementing F-6 cells which are Vero cells transformed with the HSV gH encoding gene (Fig. 2b) downstream of a HSV gD promoter, for conditional expression of gH upon infection with HSVgH-. When infected with HSVgH-, the F-6 cells produce gH which complements for the absence of gH encoding gene in the HSVgH-, therefore enabling continuous replication, in these cells. The F-6 cells are used to propagate, maintain and assay the HSVgH-.

Replication of HSVgH- :

When non-complementing cells are infected with HSVgH-, the virus particles initially possess the gH on their surface and therefore can infect the target cells. However, after the virus undergoes one complete cycle of replication, the resulting progeny virus that egress from this cell lack glycoprotein gH, and are not capable of attachment to the next target cell and hence non-infectious (Forrester A 1992). But within the cell of initial infection, the virus replicates until the cell undergoes lysis, identical to wild type HSV. Therefore the cell destruction due to infection is limited to the cell of initial infection and

the infection does not continue by spread to the surrounding cells (Fig. 3). These viruses are called Disabled Infective Single Cycle virus or DISC viruses (Sundaresan. P et al., 2000; McLean CS et al., 1994; Boursnell M.E. et al., 1997; Ali SA et al., 2000), which were originally developed as tools for vaccination against HSV-1 and for gene therapy of cancer. In the studies presented here, this property of restricted replication of the DISC virus was exploited for the purpose of inducing a limited infection in the neonatal mouse brain. It is already known that the inflammatory response may not depend on the viral replication and infection with a replication defective mutant could induce a host response comparable to a wild type HSV (McKie et al., 1998; Nguyen et al., 1992; Wood MJ et al., 1994; Wood MJ et al., 1994), which can be studied in the surviving mice.

(a)

(b)

Forrester A.H. et al., journal of Virology 1992

P_{IE-1}: Cytomegalovirus IE-1 promoter
polyA: Poly (A) addition site.

Figure 2. Constructs used to generate HSVgH- and F-6 cells

(a) LacZ encoding gene derived by BamHI digestion of pMV10. Positions of relevant restriction sites in HSV-1 DNA and HSVgH- are shown below.

(b) The 2.57 kb fragment of BstE II digested HSV-1(SC16) Bgl II-clone and Positions of the EcoRI sites in pgDBrgH, the plasmid used for transfection of Vero cells in order to generate F-6 cells.

Figure 3

Replication of HSVgH-: HSVgH- from the pools prepared in F-6 cells can enter a non-complementing cell and undergo replication, where HSV DNA and all the HSV proteins except gH. The progeny virus particles which exits the host cells lack the gH on their surface and hence non-infectious.

Other Defective Viruses used:

1. K Δ T:

K Δ T is a DISC virus with a 969 base pair deletion in the glycoprotein B (gB) gene encoding the membrane spanning region of gB in a KOS strain of HSV-1. Glycoprotein gB is essential for viral growth and has a role in infectious entry and in cell fusion and therefore a HSV with a defective gB is not infectious. Therefore K Δ T is propagated, maintained and pooled using complementing D6 cells which are Vero cells transformed with HSV gB encoding gene *in trans* and provides the virus with missing element of gB. Therefore K Δ T undergoes one cycle of replication but progeny virus particles contain a defective gB and are not infectious (Cai W et.al., 1987). This virus has the potential of causing a limited infection.

2. HSV-1 Acyclovir resistant Virus:

This strain of HSV induces mild skin lesions in SKH1 mice compared to HSV-1 (Docherty JJ et al., 2004). The virus is acyclovir resistant because the stocks were prepared in presence of 50mM acyclovir (i.e. 11.26 μ g/ml). The virus induced only mild lesions in the skin of SKH1 mice when compared to wild type HSV-1. On a scale of 5,

the maximum lesion score for acyclovir resistant HSV-1 was two while a maximum lesion score for the other HSV-1 strain was five (Docherty JJ et al., 2004).

CHAPTER 2

MATERIALS AND METHODS

Viruses and cells:

(i) SC16 Δ gH or HSVgH- is a derivative of HSV-1, strain SC16, in which the gH encoding nucleotide sequence (1110 base pairs) is replaced by a LacZ expressing sequence downstream of a cytomegalovirus IE-1 promoter, derived from plasmid pMV10 (Forrester A, et al. 1992). Because of this genomic alteration HSVgH- is able to undergo one cycle of replication in normal cells but is unable to spread to adjacent cells. Virus pools were prepared and titrated on F-6 cells which are Vero cells transformed with a plasmid containing a sequence encoding the protein gH *in trans* (Forrester A, et al., 1992), which enables the HSVgH- to replicate in a normal fashion. The HSVgH- virus and F-6 cells were a gift from Dr. Duncan Wilson (Albert Einstein Medical College, Bronx, NY), courtesy of Dr. Helena Browne (University of Cambridge, UK). The pool of HSVgH- contains a small proportion of replication competent wild type phenotype virus (approximately 1 in 200,000).

(ii) K Δ T is a derivative of KOS strain of HSV-1 with a 969 base pair deletion in a part of gB gene which includes the membrane-spanning region and a potential N-linked

glycosylation site. The K Δ T is maintained and propagated using D-6 cells which are Vero cells transformed with HSV-1 gB encoding gene *in trans*. The D-6 cells provide the K Δ T with a complete gB which complements for the defective gB encoding gene. In non-complementing cells this K Δ T can undergo only one cycle of replication, but cannot spread to the adjacent cells.

(iii) Acyclovir resistant HSV-1 is a clinical isolate of HSV-1 (0-1116) isolated from an adult brain. The stocks were prepared on Vero cells using 50 mM acyclovir (i.e. 11.26 μ g/ml). This virus was kindly provided by Dr. D. Parris and is acyclovir resistant (Parris DS et al., 1982).

Vero cells were obtained from the American Type Culture Collection (Rockville, MD) and used in viral replication studies and viral recovery assays.

F-6 cells were cultured using DMEM medium (with 5% Fetal Bovine Serum (FBS), 50 μ g/mL gentamycin and 2.25% sodium bicarbonate) and Vero cells were cultured using M-199 medium (with 5% FBS, 50 μ g/mL gentamycin and 2.25% sodium bicarbonate). Viral plaque assays were done using medium fortified with 0.5% methylcellulose for both cell lines. A laboratory strain of HSV-1 was used in plaque reduction assays.

HSVgH- replication in complementing and non-complementing cells:

The replication of HSVgH- was examined in non-complementing Vero and complementing F-6 cells. The cells were infected at a multiplicity of infection (moi) of

.01 for one hour and unattached virus was removed by washing the cells with media. The infected cells were incubated at 37° C in a 5% CO₂ humid atmosphere. At 0, 24, 48 and 72 hours post infection samples were frozen at –80° C and titered by plaque assay on F-6 cells.

Mice:

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC). Balb/C mice obtained from Charles River Laboratories (Wilmington, MA) were bred and 24-hour-old pups used in all experiments with HSVgH- virus. Balb/C mice which were <72 hours old were used for all the experiments using KΔT and Acyclovir resistant HSV-1.

Infection of neonatal mice:

Neonatal mice were cryoanesthetized in ice for 2-3 minutes and the right parietal region, just beneath the cranium injected with 0.5 µL of buffer containing 200,000, 100,000, 50,000 or 25,000 plaque forming units (pfu) of HSVgH-. Injections were performed using a Microspritzer or a Hamilton syringe with a 32-gauge needle. Uninfected Vero cells were processed and pooled in a manner identical to virus pools and used for sham infection. The mice were observed for 6 weeks in survival studies. For histological and immunohistological studies brains infected with 25,000 pfu of HSVgH- were recovered after 1, 3, 5 and 7 days of infection.

Acyclovir resistant HSV-1 was injected by a similar procedure using a Microspritizer and observed for 7 days. Infection of neonatal mice using KΔT was performed using a Microspritizer not only on the right parietal region but also in the forebrain as well as hindbrain and observed for 7-15 days.

Viral recovery from brains:

Brains were recovered 24 and 72 hours after infection and frozen at -80° C. The intact complete frozen brains were thawed and homogenized in 1mL Tris-buffered saline (TBS) and centrifuged at 10,000 rpm for 5 min. Cell debris was discarded and 50 µL of supernatant was used for plaque assays on both Vero and F-6 cells.

Assay for antibody in surviving mice:

Mice that survived infection after intracerebral injection with 25,000 pfu were bled by cardiac puncture 6 weeks post infection, sera recovered and assayed for anti-HSV antibody by a plaque reduction assay. The serum from four different mice was serially diluted and incubated with 200 pfu of HSV-1 at 37° C for one hour. Controls included anti-HSV-1 antiserum, normal mouse serum and TBS (Tris Buffered Saline, pH 7.4). At the end of one hour the samples were titrated by plaque assay on Vero cells.

Immunohistology:

Brains were fixed in 10% neutral buffered formalin for 6 hours, processed in a Histoprocessor and embedded in paraffin. Sections 20 μ M thick were used when staining for microglia and 6 μ M thick sections were used when staining for astrocytes or T and B cells.

Microglia: Tissue sections were rehydrated, trypsinized for 10 minutes (Hauke C et al., 1993) and incubated with biotinylated Ricinus Communis agglutinin-I (RCA-I; Vector Labs) which binds to β -d-galactose residues on microglia, for one hour. Sections were washed 3 times for 5 minutes each in phosphate buffered saline (PBS) containing 0.1% TritonX-100. The sections were then incubated with Streptavidin–Horseradish peroxidase (HRP) (Jackson ImmunoResearch) for 30 minutes followed by a PBS rinse and a Diaminobenzidine (DAB) (Sigma-Aldrich) disclosing reaction. Sections were counterstained using hematoxylin and dehydrated in increasing concentrations of ethanol then xylene.

Astrocytes: Tissue sections were incubated with Rabbit anti-GFAP antibody (Santa Cruz Biotech) for 2 hours, followed by 3 washes of 5 minutes each in PBS and detection using DAKO EnVision+ system with DAB. Sections were counterstained with hematoxylin and dehydrated in increasing concentrations of ethanol then xylene.

T and B Cells: Tissue sections were incubated with Rabbit anti-CD3 antibody for T cell detection and Rabbit anti-CD20 antibody for B cell detection (Santa Cruz Biotech) after antigen retrieval using Trilogy antigen retrieval solution (Cell Marque). The same procedure described above for detection of astrocytes was performed. Adult mouse spleen sections were used as positive controls.

Detection of cells infected with HSVgH-: Immunoperoxidase staining was performed on adjacent paraffin embedded sections of 6µM thickness using rabbit polyclonal anti-HSV antibody (Biomeda) and monoclonal anti-β-galactosidase antibody (Cappel). The same procedure described for T and B cell staining was used except for overnight incubation with the primary antibody at 4° C.

LacZ staining:

HSVgH- infected brains were fixed in 4% p-formaldehyde and whole brain mounts were stained overnight using a LacZ staining solution containing X-gal (Sigma Aldrich), potassium ferrocyanide, potassium ferricyanide and magnesium chloride (Fisher Scientific). The LacZ stained brains were frozen, 50 µM thick sections prepared and counterstained with neutral red.

Detection of Apoptotic cells:

Detection of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) positive apoptotic cells in infected neonatal brains was done on paraffin

sections using ApopTag peroxidase in situ apoptosis detection kit (Chemicon International) as described by the manufacturer and counterstained with hematoxylin. At 1, 3, 5 and 7 days post infection sections adjacent to the HSV antigen positive sections were examined.

CHAPTER 3:

RESULTS

Generation of a circumscribed HSV-1 lesion in vivo:

1. Experiments with KΔT virus:

In initial studies neonatal mice <72 hours old were infected with KΔT by intracerebral injection of 0.5μL of inoculum containing 200,000, 100,000, 20,000, 10,000, 8000, 5000, 2000 or 1000 pfu of KΔT using a microspritizer. The pools of KΔT virus used also contained wild type (WT) virus in a ratio of approximately 1 WT for every 1666 mutant viruses. The infected mice were observed for a period of 7 days. The results showed mortality rate directly proportional to the amount of infecting virus. Survival rates ranged from 60-100% when 2000 pfu of KΔT or less was used to infect the mice. When using 5000 pfu or greater of KΔT survival rates were less than 30% (Fig. 4). Although infection with 2000 pfu resulted in high survival rates, it would likely not be sufficient to cause a sizeable lesion required for our studies. Therefore we attempted to reduce or eliminate the mortality observed after infection using higher number of pfu's.

Figure 4. Survival study of neonatal mice infected with KΔT virus : Neonatal mice were infected with specified amounts of KΔT virus by intracerebral injections (forebrain/midbrain/hindbrain on the right side) and observed for 7 days post infection. The wild type virus mixture present in the inoculum is also indicated. n= number of mice used.

At this point it was not certain whether it was the wild type virus present in the pools or combination of KΔT and the wild type virus, which was responsible for the observed mortality. Therefore we attempted to control the infection and reduce the mortality by acyclovir treatment of infected mice. Because intravenous acyclovir is not practical in case of the neonatal mice other routes of administration were used.

In the next set of experiments neonatal mice were infected with 20,000 pfu of KΔT virus, which contains 12 pfu of WT virus, a combination shown earlier to cause 100% mortality. They were treated once a day subcutaneous with acyclovir (10mg/kg body weight) starting at 48 hours post infection for three days. Results showed that this treatment delayed the mortality by two days but did not reduce mortality relative to controls (Fig.5). Subcutaneous acyclovir was not effective in reducing mortality of infected neonatal mice.

The next set of experiments was designed to deliver a more intensive acyclovir treatment to the infected neonatal mice. For this purpose neonatal mice were given twice a day injections with acyclovir by intraperitoneal route of administration starting at 24 hours post infection. Also at 24 hours post infection the mother was injected intraperitoneally with acyclovir (500μL) twice a day, since acyclovir secreted in breast milk further enhances the bioavailability in the infected neonatal mice (Fig. 6). However there was only a 10% increase of survival in spite of this intensive treatment.

Figure 5. Survival study of neonatal mice infected with KΔT virus and treated with subcutaneous Acyclovir: Neonatal mice were infected by intracerebral injection on the right side of the midbrain with 2×10^4 pfu of KΔT (containing 12 pfu of WT). They were treated once a day with 100 μ L of acyclovir (10 mg/kg) by subcutaneous injection at 48, 72 and 96 hours post infection. Untreated infected mice were used as controls. n= number of mice used.

Figure 6. Survival study of neonatal mice infected with KΔT virus and treated with intensive Acyclovir treatment: Neonatal mice were infected by intracerebral injection on the right side of the midbrain with 2×10^4 pfu of KΔT (containing 12 pfu of WT) and treated twice a day with acyclovir $4 \mu\text{g}/10 \mu\text{L}$ ($50 \mu\text{L}$) by intraperitoneal route starting at 24 hours post infection. Also at 24 hours post infection mother was treated with $500 \mu\text{L}$ acyclovir ip twice a day. The infected neonatal mice were observed for 14 days post infection. n= number of mice used.

It is well known that HSV-1 when infected intracerebrally with small amounts of virus can cause high mortality in neonatal animals because of its unlimited replication. Studies have shown that HSV-1 virus in amounts as low as 1 pfu, when injected intracerebrally into neonatal rats can cause significant mortality (Engel JA et al., 2000). Since the amount of WT virus present in the inoculum we used was demonstrable, we reasoned that the high mortality of the infected neonatal mice might be due to the uncontrolled replication of WT virus present in the virus pool. Therefore the next set of experiments was designed to test this hypothesis.

WT virus present in the KΔT virus pool was amplified by culturing the virus pool mixture on Vero cells only. Vero cells permit only one cycle of replication of KΔT virus whereas continuous replication of WT virus and would therefore select for WT virus. Thus after three serial passages only the WT virus were selected which was confirmed by similar titers on plaque assays in Vero and D-6 cells. Neonatal mice were infected intracerebrally as described earlier using 5, 15 and 50 pfu of the selected WT virus and observed over a period of 2 weeks (Fig.7). These infections resulted in very high mortality starting from day 1 post infection and reaching 100% among the mice infected with 15 and 50 pfu by 5 and 6 days post infection respectively. Whereas in case of mice infected with 5 pfu, mortality was observed at 1 day post infection but reached 90% at 9 days post infection.

In most of our experiments the amount of virus used to infect the neonatal mice contained more than 5 pfu of WT viruses suggesting that the high mortality in case of intracerebral infection with K Δ T virus was most likely due to the wild type virus present in the mixture and not the mutant K Δ T. In our studies infection with this virus resulted in high mortality of the infected neonatal mice, which was not reduced by treatment with acyclovir. So a non-lethal neonatal mouse model of controlled HSV-1 encephalitis could not be achieved using K Δ T virus, and hence alternative defective HSV-1 derivatives were considered.

Figure 7. Survival study of neonatal mice infected with WT virus selected from KΔT pools: Neonatal mice were infected intracerebrally (right midbrain) with 0.5μL of buffer containing 5, 15 and 50 pfu of pooled WT selected from the KΔT virus pool by serial passage on Vero cells. The infected mice were observed for 15 days post infection. n= number of mice used.

2. *Experiments with Acyclovir resistant HSV-1:*

Neonatal mice <72 hours old were infected with 5, 15 and 50 pfu of acyclovir resistant HSV-1 on the right parietal region, using a microspritizer and observed for survival. Regardless of the amount of virus used, mortality was observed from day 2 post infection. Among the mice infected with 15 and 50 pfu, there was 100% mortality by 4 days post infection, whereas infection using 5 pfu resulted in 100% mortality by day 6 post infection (Fig. 7). Intracerebral inoculation in neonatal mice using acyclovir resistant HSV-1 resulted in rapid mortality, in spite of using small amounts of virus and therefore was not suitable for achieving a non-lethal infection. Therefore another defective HSV-1 was tested.

Figure 8. Survival study of neonatal mice infected with acyclovir resistant HSV-1: Neonatal mice <72 hours old were infected with 5, 15 or 50 pfu of Acyclovir resistant HSV-1 by intracerebral inoculation and observed for survival. n= number of mice used.

3. *Experiments with HSVgH-:*

HSVgH- is a DISC virus having properties similar to KΔT, but with some distinct advantages. The stocks contained fewer wild type virus (1 in 200,000) which would enable us to use higher amounts of mutant virus but lesser amount of background wild type virus particles. HSVgH- also produces β -galactosidase in the infected cells which makes detection of this virus easier.

In vitro studies:

(i) Viral replication studies-Quantitative:

In order to develop a model of focal lesion using a DISC virus, studies verifying that the HSVgH- undergoes a single cycle of replication in non-complementing cells were undertaken. A growth study was carried out on non-complementing Vero cells and complementing F-6 cells. These cells were infected with the virus at a moi of .01 for one hour and at 24, 48 and 72 hours post infection samples were collected and titered by the plaque assay on F-6 cells. The results (Fig.9) show that no infectious HSVgH- was detected at 48 hours post infection in non-complementing Vero cells, indicating that the virus was not able to undergo continuing rounds of replication. Whereas in complementing F-6 cells the amount of virus produced increased for 48 hours post infection before achieving a plateau, indicating that the virus underwent multiple rounds

of replication. These results verify that the HSVgH- undergoes a single cycle of replication in non-complementing gH- cells.

Figure 9. Replication of HSVgH- in non-complementing Vero cells and complementing F-6 cells: F-6 and Vero cells were infected with HSVgH- at a moi of 0.01 (1 pfu of HSVgH- for every 100 cells plated), cells frozen at 0, 24, 48 and 72 hours post infections which were titrated by plaque assays on F-6 cells.

(ii) Viral replication studies-Qualitative:

Further studies were carried out to study the replication of HSVgH-. F-6 and Vero cells were infected with HSVgH- at a moi of 0.1. At 24 hours post infection the cells were fixed and stained to detect infected cells by immunohistochemistry using specific polyclonal antibody for the HSV-1 antigens. Since the virus contains a lacZ encoding gene, β -galactosidase is expressed in the infected cells, which were alternatively detected in a different set of experiments by X-gal staining. Results show that at 24 hours post infection in F-6 cells the positively stained or infected cells were seen in clusters (Fig.10 a and 11a) indicating that the virus was able to infect surrounding cells during subsequent rounds of infection in complementing cells. In contrast in Vero cells (Fig 10b and 11b) the infected cells were dispersed and not seen in clusters, indicating that the inability of virus to spread and infect the surrounding cells and undergo more than one cycle of replication in non-complementing cells.

These results confirm that HSVgH- infection results in a local limited infection in vitro in non-complementing cells. The limited capacity of the HSVgH- to cause infection will be utilized in vivo to create a limited lesion.

Figure 10. Immunoperoxidase staining showing HSVgH- infection in vitro:

Immunoperoxidase stained F-6 (a) and Vero (b) cells which were infected with HSVgH- at an moi of 0.1 fixed at 24 hours post infection. The arrows indicate cluster of infected cells in (a) and isolated infected cells in (b).

Figure 11.: LacZ staining showing HSVgH- infection in vitro: LacZ stained sections of F-6 (a) and Vero (b) cells which were infected with HSVgH- at an moi of 0.1 stained with X-gal staining solution at 24 hours post infection. The blue cells are infected and positive for LacZ, the spaces in between contain uninfected cells.

INVIVO STUDIES:

(i) SURVIVAL STUDIES:

In order to determine the quantity of HSVgH⁻ virus required to cause a detectable but nonlethal CNS infection, neonatal Balb/C mice were inoculated in the right parietal region and observed for survival for 6 weeks post infection. Results (Figure 12) showed that infection with 200,000, 100,000 and 50,000 plaque forming units (pfu) resulted in survival of 0%, 22%, and 41% of infected mice, respectively. However, infection with 25,000 pfu resulted in 75.5% survival. When the mice died, 98% of the deaths occurred between 3 and 7 days post infection, regardless of the amount of virus used. Mice that survived this period went on to survive for at least 6 weeks, the end point of the study. Kaplan-Meier survival analysis revealed a high statistical significance when tested for the difference between survival patterns with different amounts of virus ($P < .001$). In the remaining studies, 25,000 pfu were used because a sufficient number of infected neonatal mice survived for use in the described experiments.

Figure 12. Survival study of neonatal mice infected with HSVgH-: Kaplan-Meier survival analyses of 24-hour-old neonatal mice infected with 200,000, 100,000, 50,000 and 25,000 pfu of HSVgH- virus and observed for 6 weeks post infection. Experiments using 50,000 and 25,000 pfu of virus were carried out twice. (All four groups were compared using Gehan-Breslow test; $P < .001$).

(ii) Viral recovery from infected brains

As previously mentioned, the virus pools used contained wild-type virus at a ratio of approximately 1:200,000. Because wild-type virus can undergo uncontrolled replication, it would explain the observed mortality when injecting higher quantities of virus. To detect the presence of wild-type HSV in the infected neonatal mice, viral recovery experiments were performed using infected brains to recover replication competent viruses. The results were interpreted on basis of titers on Vero and F-6 cells.

Once mutant virus replicates in the brain, it cannot form plaques on Vero cells or F-6 cells. But if any of the infected mice received wild type virus, they should form plaques on Vero as well as F-6 cells and also have comparable titers on both these cells.

Therefore any plaques observed on Vero cells or F-6 cells would be due to virus with a wild-type phenotype.

(a) Viral recovery from neonatal mice infected with 25,000 pfu of HSVgH-:

In neonatal mice infected with 25,000 pfu, 2 out of 17 (11.7%) of the mice had recoverable wild-type virus 1 day after infection (Figure 13a), indicated by similar titers on F-6 and Vero cells. When brains of mice infected for 3 days were examined, wild-type virus was recovered from 4 of 13 mice (30.7%; Figure 13b). Animals were not examined on days 5 and 7 because some expired after day 3 and the condition of the tissue precluded efficient viral recovery. Collectively over a 3-day period, 20% (6/30) had recoverable wild-type virus. The results of viral recovery experiments roughly

correspond with the death rate of the infected neonatal mice in the survival studies (Figure 12) infected with the same amount of virus.

Figure 13. Viral recovery from neonatal mice brains infected with 25,000 pfu of HSVgH-: Replication competent viruses recovered by plaque assays using infected mice brain extracts. **(a)** At 1 day post infection a total of 17 mice were tested, mice 5 and 15 were positive. **(b)** At 3 days post infection 13 mice were tested, mice 2, 6, 10, and 13 were positive.

(b) Viral recovery from neonatal mice infected with 50,000 pfu of HSVgH-:

In order to test the hypothesis further, viral recovery was attempted using brain extracts of neonatal mice infected with 50,000 pfu of HSVgH-. In this experiment, 5 out of 13 (38.46%) of the mice had recoverable wild-type virus 1 day after infection (Figure 14a), indicated by similar titers on F-6 and Vero cells. When brains of mice infected for 3 days were examined, wild-type virus was recovered from 5 of 11 mice (45.45%; Figure 14b). Collectively over a 3-day period, ~42% (10/24) had recoverable wild-type virus. These results combined with the results of viral recovery from neonatal mice infected with 25,000 pfu and the survival studies further support the possibility that the observed mortality is entirely due to the wild type HSV.

Figure 14. Viral recovery from neonatal mice brains infected with 50,000 pfu of HSVgH-: Replication competent viruses recovered by plaque assays using infected mice brain extracts. **(a)** At 1 day post infection a total of 13 mice were tested, mice 2, 4, 6, 7 and 10 were positive. **(b)** At 3 days post infection 11 mice were tested, mice 1, 5, 7, 8 and 11 were positive.

(iii) In vivo demonstration of HSVgH- infection:

Brains were recovered from neonatal mice infected with 25,000 pfu of HSVgH- 1, 3, 5, and 7 days post infection and examined for LacZ. Whole brains were processed and stained for LacZ and frozen sections of brains were counterstained with neutral red. LacZ positive cells were detected in brain sections (Table 1, Figure 15 and 16) at the site of injection at day 1 post infection in all brains. At days 3, 5, and 7 post infection, LacZ positive cells were not detectable (Table 1), although signs of cerebral edema and hydrocephalus consistent with an infection were evident. To confirm the presence of virus-infected cells in the brains, paraffin-embedded sections of infected brains were stained with anti-HSV primary antibody. Adjacent sections were stained with anti- β -galactosidase primary antibody.

Results showed the presence of HSV antigen-positive cells at 1 and 3 days post infection (Table 1 and Figure 17a, 18a), but not at 5 and 7 days post infection. β -galactosidase-positive cells were observed on day 1 post infection in sections adjacent to HSV antigen-positive sections (Table 1 and Figure 17b), but they were not detected at 3, 5, and 7 days post infection. Although the infection was mostly limited to the site of injection, HSV- and β -galactosidase-positive cells were occasionally detected in other areas, including deep cerebral cortex, white matter, and ependymal lining, likely due to the nonstereotactic nature of the injection. These results confirm the presence of HSVgH- infection in the neonatal mouse brain infected with 25,000 pfu of virus by intracerebral inoculation.

Table 1 Detection of infected and apoptotic cells

	<i>Days post infection (positive/total)</i>			
	<i>1</i>	<i>3</i>	<i>5</i>	<i>7</i>
LacZ (x-gal histochemistry)	4/4	0/4	0/5	0/7
Anti- β -galactosidase antibody	3/3	0/3	0/3	0/3
Anti-HSV antibody	3/3	3/3	0/3	0/3
TUNEL staining	0/4	4/4	0/4	0/4

Table 1. Detection of cells positive for β -galactosidase (X-gal histochemistry and immunoperoxidase staining using anti- β -galactosidase antibody), HSV (immunoperoxidase staining using anti-HSV antibody), and apoptosis (TUNEL) in infected neonatal mice at 1, 3, 5, and 7 days. X-gal histochemistry was performed on whole brains and frozen sections prepared from these brains were counterstained with neutral red. Paraffin sections were used for immunoperoxidase staining as well as TUNEL staining.

Figure 15. Infected neonatal mouse brain stained using X-gal histochemistry: Whole brain extracted from a 24-hour-old Balb/C mouse infected with 25,000 pfu of HSVgH-, 1 day post infection and stained using X-gal histochemistry, demonstrating the blue-colored infected area

Figure 16. Frozen histological section of a neonatal mouse showing HSVgH-infected cells : Frozen section of brain extracted from a 24-hour-old Balb/C mouse infected with 25,000 pfu of HSVgH-, 1 day post infection and stained using X-gal histochemistry and counterstained with neutral red. The blue cells are positive for X-gal as well as HSVgH-.

Figure 17. Immunoperoxidase-stained paraffin-embedded brain sections at 1 day post infection. (a) Section stained using anti-HSV antibody showing HSV-positive cells (*brown*). (b) Adjacent section stained using anti- β -galactosidase primary antibody showing β -galactosidase-positive cells (*brown*). Sections were counterstained using hematoxylin.

(iv) Detection of Apoptotic cells:

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) staining was performed using paraffin-embedded sections to detect presence of apoptotic cells. In brains that were examined 1 and 3 days post infection, sections adjacent to HSV antigen-positive sections were used and in brains that were examined 5 and 7 days post infection, one in every five serial sections were used for TUNEL staining. At day 1 post infection, TUNEL-positive cells were not detected, although HSV antigens were detected (Table 1). At 3 days post infection, TUNEL-positive cells were detected in all the examined brains, in the same areas where HSV antigens were detected (Figure 18 a, b and Table 1). At 5 and 7 days post infection, TUNEL-positive cells were not detected (Table 1).

Immunoperoxidase staining using anti-HSV Ab

TUNEL staining

Figure 18. Detection of apoptosis in infected neonatal brains: Adjacent brain sections at 3 days post infection. (a) Immunoperoxidase-stained section showing cells positive for HSV antigens (*brown*) and (b) TUNEL-stained section showing cells positive for apoptosis (*brown*) in the infected area shown in (a). The sections were counterstained using hematoxylin.

(v) *Lack of neutralizing antibody in surviving mice:* In order to determine if neutralizing antibodies are formed in mice that survived infection with 25,000 pfu of HSVgH-, a plaque reduction assay was performed using sera from four mice, six weeks after they were intracerebrally injected with HSVgH-. The serum from each mouse was independently tested for neutralizing antibodies and the data then combined. Results showed that there was no reduction in the number of plaques (Figure 19) indicating a lack of HSV neutralizing antibody in the serum of these mice. Incubation with the positive control goat anti-HSV antibody, showed 100% neutralization of the virus and no neutralization using negative controls of normal mouse serum or buffer. These studies indicate that intracerebral infection with 25,000 pfu of HSVgH- virus did not result in the formation of detectable neutralizing antibodies measured at 6 weeks post-infection.

Figure 19. Plaque reduction assay to detect the presence of neutralizing anti-HSV antibodies: Serum from 4 mice was collected 6 weeks after intracerebral infection and each incubated with 200 pfu of HSV-1 for one hour at 37 °C. Serum dilutions were 1:4, 1:8 and 1:16. Samples were titrated on Vero cells by plaque assay. Goat anti-HSV antibody, TBS and normal mouse serum (1:4 dilution) were included as controls. SEM of results from sera of 4 mice are represented.

(vi) Host response

In order to characterize early host cellular response to the presence of intracerebral HSVgH⁻, virus-infected and sham-infected brains were examined for the presence or absence of astrocytes, microglia, and T and B lymphocytes at days 1, 3, 5, and 7 post infection by immunohistochemistry.

Microglial activation was evident from the RCA-I-positive staining in the white matter of infected brains on day 1, 3, 5, and 7 post infection (Table 2). At 7 days post infection, numerous microglia were detected in the cerebral cortex, around the site of inoculation (Figure 20a). Microglia in the resting state characterized by smaller cell bodies and long slender processes, as well as activated state characterized by larger cell bodies with short stumpy processes, were detected in infected brains. In sham-infected brains a few resting microglia were observed and no activated microglia were seen (Figure 20b).

Staining with anti-GFAP primary antibodies showed astrocytic proliferation in the cortex as well as white matter beginning primarily on day 3 post infection (Figure 21a) and persisting on day 7 post infection (Table 2). In contrast, in sham-infected brains no GFAP-positive cells were detected at any of the time points tested (Figure 21b).

CD3- and CD20-specific primary antibodies were used to test for the presence of B and T lymphocytes, respectively. B(Fig 22) and T lymphocytes (Fig 23) were readily

detected in control tissue of adult mouse spleen. However, neither T cells nor B cells were detected in virus-infected or sham-infected neonatal mice brains throughout the 7 days that brain sections were examined (Table 2).

Table 2 Host Response

Cell types (markers)	1 dpi (Positive/Total)		3 dpi (Positive/Total)		5 dpi (Positive/Total)		7 dpi (Positive/Total)	
	<i>infected</i>	<i>sham infected</i>	<i>infected</i>	<i>sham infected</i>	<i>infected</i>	<i>sham infected</i>	<i>infected</i>	<i>sham infected</i>
Microglia (RCA-I)	4/4	0/4	4/4	0/4	6/6	0/3	5/5	0/3
Astrocytes (GFAP)	1/4	0/2	4/4	0/2	4/4	0/3	4/4	0/3
T lymphocytes (CD3)	0/4	0/2	0/4	0/2	0/4	0/3	0/4	0/3
B lymphocytes (CD20)	0/4	0/2	0/4	0/2	0/4	0/3	0/4	0/3

Table 2. 24-hour-old mice were infected with 25,000 pfu of HSVgH- virus or sham infected with Vero cell extracts by intracerebral injection. Brains were extracted from the infected mice at 1, 3, 5, and 7 days post infection (dpi) and paraffin sections were used to stain for specific cell types.

Figure 20. Microglial proliferation at 7 days post infection in the cerebral cortex of a mouse brain infected when it was 24 hours old with 25,000 pfu of HSVgH⁻. Paraffin sections were stained using RCA-I. (a) Infected mouse brain showing numerous positively stained microglia (*brown*) in the cortex and (b) Sham-infected mouse brain showing few microglia.

Figure 21. Astrocytosis at 3 days post infection in the cerebral cortex of a mouse brain infected when it was 24 hours old, with 25,000 pfu of HSVgH⁻. Paraffin sections were stained using anti-GFAP primary antibody. **(a)** Infected mouse brain showing numerous positively stained astrocytes (*brown*) in the cortex and **(b)** Sham-infected mouse brain showing no GFAP positive astrocytes

(a) Adult mouse spleen (positive control)

(b) Infected brain (80x)

(c) Sham Infected brain (80x)

Figure 22. B-lymphocytes not detected in the infected brain : B-lymphocytes were not detected in the HSVgH- infected brains at 3 days post infection. Paraffin sections were stained using anti-CD20 primary antibody and counterstained using hematoxylin. (a) Adult mouse spleen used as positive control showing CD-20 positive B-lymphocytes (brown). No CD-20 positive B-Lymphocytes were detected in (b) Infected mouse brain and (c) Sham infected mouse brain.

(a) Adult mouse spleen (positive control)

(b) Infected brain (80x)

(c) Sham Infected brain (80x)

Figure 23: T-lymphocytes not detected in the infected brain: T-lymphocytes were not detected in the HSVgH- infected brains at 3 days post infection. Paraffin sections were stained using anti-CD3 primary antibody and counterstained using hematoxylin. **(a)** Adult mouse spleen used as positive control showing CD-3 positive T-lymphocytes (brown). No CD-3 positive T-Lymphocytes were detected in **(b)** Infected mouse brain and **(c)** Sham infected mouse brain.

CHAPTER 4:

DISCUSSION

HSV encephalitis in a neonate usually results in hemorrhagic necrosis leading to widespread tissue destruction and rapid mortality. Because of the rapid lethality of HSV encephalitis, characterization of this disease has been limited. In an attempt to circumvent this limitation, studies described here utilized a defective HSV which would result in a limited controlled infection which would not cause mortality and permit the study of the initial stages of infection.

The K Δ T virus is a defective HSV which would infect a cell and undergo one cycle of replication, but the progeny viruses lack the surface glycoprotein gB and are no longer infectious (Cai WL et al., 1987). Therefore intracerebral infection of neonatal mice with this virus should ideally result in a limited HSV infection. But when the viral stocks were assayed on the complementing D6 and non complementing Vero cells it was evident that there was a high amount of virus with wild type phenotype.

When higher amounts of KΔT were used to infect the mice, it invariably resulted in near total mortality. Because of the presence of background wild type virus (1 in 1666), the inoculum contained wild type virus in significant numbers and therefore resulted in high mortality. The mortality was reduced when lower amounts of KΔT were used because the background wild type virus particles in the inoculum were reduced to negligible levels. However such an infection is unlikely cause a detectable lesion.

In the next set of experiments infected mice were treated with subcutaneous acyclovir in order to control the infection. However treatment only delayed mortality and did not reduce it. In neonatal herpes infections, intravenous acyclovir is the treatment of choice, which is not practical in case of a neonatal mouse. Bioavailability of acyclovir with subcutaneous injections was probably not sufficient to control the HSV infection. A more intensive treatment was undertaken by intraperitoneal acyclovir administration in the infected neonatal mice. To enhance the bioavailability of acyclovir in the infected mice, the nursing mother was also administered with acyclovir because since acyclovir is secreted in the breast milk. In spite of this therapy, the survival rate did not show any significant improvement.

At this time the role of the wild type phenotype virus present in the KΔT pools in the observed mortality was not clear. It appeared that the wild type virus alone caused all the mortality. Therefore to test this hypothesis, the wild type virus was extracted from the KΔT pools by serial culture on Vero cells. Vero cells permit one cycle of replication of KΔT virus but continuous replication of the wild type virus and hence serial passage in

Vero cells selects for wild type virus. Infection of neonatal mice using as low as 5, 15 and 50 pfu of this wild type virus resulted in 100% mortality. Since this wild type virus was present in significant numbers, the mortality observed during the survival studies using KΔT was very likely due to the wild type virus alone. It was difficult to minimize the number of this wild type virus in the inoculum since the mutant virus when used to infect in very low quantities might not cause a detectable lesion. Therefore we were not successful in inducing a non-lethal HSV infection in neonatal mice using KΔT.

The acyclovir resistant HSV-1 (Parris DS et al., 1982) is a poorly characterized clinical isolate known to cause minimal lesions in the skin infection model in SKH1 mice (Docherty JJ et al., 2004). This virus unlike the KΔT is capable of continuous replication. Provided that the virus demonstrated virulence similar to the skin lesion model in SKH1 mice, this virus should cause a mild CNS lesion in the neonatal mice. Therefore attempts were made to determine the neurovirulence of this virus in neonatal mice. However intracerebral inoculation of this virus in neonatal mice resulted in rapid total mortality. The acyclovir resistant HSV-1 demonstrated higher neurovirulence compared to the wild type phenotype HSV selected from the KΔT virus pools. This property of the acyclovir resistant HSV-1 may be the result of a higher susceptibility of the CNS per se or the neonatal CNS in particular. Therefore we were not successful in inducing a non-lethal HSV infection in neonatal mice using acyclovir resistant HSV-1.

HSVgH- is another DISC virus (Forrester A et al., 1992), very similar to KΔT in many respects, but with distinct advantages. Although the HSVgH- pools also contained wild type virus, their proportion was low compared to the KΔT virus pools. This allowed infection using fairly high amounts of HSVgH- containing negligible amounts of wild type virus present in the inoculum. An additional advantage is that the HSVgH- produces β -galactosidase in the infected cells, and hence enables easier detection of the infected cells. Using this virus an experimental infection in the neonatal mouse brain was generated that resulted in a detectable but limited lesion. The mortality rate was low enough to permit us to study the host response to the disease over the first seven days of infection.

In a series of experiments it was determined that intracerebral injection with 200,000 pfu of HSVgH- resulted in 100% mortality. But when 25,000 pfu of this virus was used approximately 75% of the mice survived. Because the mutant virus pool contained a small proportion of wild type virus (1 in 200,000), it is possible that it was responsible for the observed mortality since the larger inoculum would have contained more wild type virus. In this regard earlier studies reported that intracerebral infection of neonatal rats with wild type HSV-1 in amounts as low as 2.5 pfu resulted in 60% mortality and 0.6 pfu of HSV-1 resulted in 40% mortality (Engel J A et al., 2000). In addition replication competent virus with a wild type phenotype was recovered from 20% of mice infected with 25,000 pfu and 42% of the mice infected with 50,000 pfu (data not shown). However, other possibilities to account for mortality in these mice which cannot

be excluded include immunopathogenic host response to the presence of more viral antigens and transneuronal spread of the virus by synaptic transmission. Based on these results we concluded that 25,000 pfu of HSVgH- was the optimal dose required to limit mortality, while preserving sufficient numbers of infected Balb/C neonates to study the early events of encephalitis.

Viral presence in the brains of infected mice was established by detecting LacZ positive cells and cells containing viral antigens. LacZ positive cells were detected only on day 1 post infection, and not at days 3, 5 and 7-post infection. This is not surprising since LacZ expression in the infected cells is limited to early stages of infection since it is driven by the cytomegalovirus IE-1 promoter, which is likely to be turned off after initial stages of infection. Also the presence of virus was established by the demonstration of HSV antigen positive cells detected on 1 and 3 days post infection. In addition cresyl violet and hematoxylin/eosin stained sections revealed cellular changes and responses consistent with HSV pathology including polymorphonuclear infiltration, pallor of staining and loss of cells at the infected site. At 5 and 7 days post infection pale staining and signs of hydrocephalus which indicate HSV infection were observed, although no HSV or Lac Z positive cells were observed.

Classically herpes encephalitis has been described as necrotizing encephalitis; however the severity of histopathologic changes and neurological symptoms does not correlate with the viral burden in the brain, indicating other mechanisms are involved in pathogenesis. Apoptosis is a mechanism of viral induced cell death in infected neuronal

cell cultures in vitro and experimental models of central nervous system in vivo infections (Griffin DE et al.,1999; Allsopp TE et al.,2000). Many neurotrophic viruses have been shown to induce neuronal and glial apoptosis (Depres P et al.,1998; Liao CL et al., 2001; Pokosz A et al.,1996; Matthews et al., V 2000; Schoneboom BA et al.,2000; Jackson AC et al.1997; Parquet MC et al. 2001; Weissenbock H et al. 2000) including herpes virus (Geiger KD et al.,1997; DeBiasi RL et al., 2002; Shaw MW et al. 2001). Although HSV infection has been directly implicated in multiple studies, mechanism of cellular and tissue injury remains poorly understood. But there is sufficient evidence which points to induction of apoptosis by HSV-1 during the initial phase, and in presence of a total protein synthesis inhibitor the virus is unable to prevent apoptosis, but the later viral or cellular products inhibit apoptosis (Koyama AH et al., 1997; Goodkin ML et al., 2004). But the question would be in what way apoptosis would benefit the host?

Inhibition of apoptosis in order to prolong the life of the cell would benefit the virus replication and apoptosis is considered one of the non-specific host responses to acute HSV infection that may aid the host by limiting replication and spread of the virus (Irie H et al., 2004). Previous studies have described apoptotic cell death of infected cells and the bystanders in adult mouse brain (Shaw M M et al., 2002). The results of the study presented here is consistent with these studies since TUNEL positive cells were detected in HSVgH- positive areas, in sections adjacent to the HSVgH- positive sections at 3 days post infection. TUNEL positive cells however were not detected on days 5 and 7 post infection. Apoptotic cells are known to be cleared rapidly in vivo (Johnson et al.,1997)

therefore it is possible that the cells at day 3 post infection that were TUNEL positive rapidly died and were cleared, therefore not detectable at 5 and 7 days post infection. Also because progeny virus was non-infectious they were unable to infect adjacent cells leading to the presence of TUNEL positive cells on day 5 and 7 post infection.

The host cellular response to the presence of HSV in the neonatal brain was examined. Astrogliosis characterized by GFAP- immunoreactivity is a typical response seen in case of injury and inflammation to the adult CNS (Latov N et al., 1979; Smith ME et al., 1983; Mathewson and Berry 1985). The CNS injury may be due to mechanical trauma, neuronal necrosis, ionic changes (Mathewson AJ and Berry M, 1985) or may be a result of entry of inflammatory cells into the CNS when the blood brain barrier is disrupted. In neonatal animals, studies have shown that astrocytic response to CNS injury is absent or minimal (Bignami A and Dahl D, 1976; Gearhart J et al., 1979; Bernstein DR et al., 1981; Maxwell WL et al., 1990). It has been suggested that in adult animals the observed astrocytic response is mainly a result of cytokine production, which is lacking in the neonatal animal. However this concept has been challenged by the results from other studies where extensive astrogliosis was observed in response to intracerebral nitrocellulose implantation (Balasingham V et al., 1994). These investigators concluded that astrogliosis can occur in the neonatal brain if a sufficient stimulus is present (Balasingham V et al., 1994) and inflammatory cytokines are elevated not only in adult animals but also in neonatal animals, associated with astrogliosis (Rostworowski M et al., 1997). In agreement with these studies the results presented here showed astrocytic

proliferation in the cerebral cortex at the site of infection at 3, 5, and 7 days post infection.

Continuing the studies of the non-specific cellular response to the presence of HSV microglial response was examined. Microglial activation is also a known result of cytokine release (Chan A et al., 2003). Microglia and macrophages are thought to play an important role in the immune response in viral encephalitis (Esiri MM and Kennedy, 1992). In the studies presented here microglial activation in the white matter was the earliest observed response to HSVgH- infection starting on day 1 post infection. By 7 days post infection, both resting and activated microglia were observed in the cerebral cortex, at or near the site of injection. In contrast, microglia in sham-infected brains were barely detectable by RCA-I staining and these were only the resting variety.

In the next experiments the adaptive cellular response in the neonatal brain to HSV was examined. Notably in these studies T- and B-lymphocytes were not detectable by immunoperoxidase staining using anti-CD3 and anti-CD20 antibodies during the first seven days of infection. Earlier studies with adult Balb/C mice have reported recruitment of T cells but not B cells in the brain after intracerebral inoculation with HSV-1 (McKie et al., 1998). Other studies in neonatal mice infected with herpes viruses at different sites resulted in failure of activation of lymphocytes, most likely because of an immature immune system (Kohl S et al., 1982). This latter observation is consistent with the results presented here of a lack of T and B-cell response in the infected brains. However that does not exclude the possibility that in the system used here the lack of adaptive response

is merely a reflection of limited quantities of viral antigen in the neonatal brain. The adaptive response of the neonate to the HSVgH- (a DISC virus) used in these studies may be quite different than to a wild type virus.

As mentioned earlier, the extensive astrogliosis may be a result of cytokine release. But in the studies presented here no lymphocytes were detectable, although neurons, microglia and astrocytes themselves (Guilian D et al., 1987; Logan A et al., 1992; Wesselingh SL et al., 1993) are thought to produce cytokines. Cell death resulting in cytokine release causing initial activation of microglia and astrocytes, followed by an enhanced response resulting from cytokine release from these cells may be a possible mechanism.

In conclusion, the results show that CNS inoculation with HSVgH- in sufficient amounts results in an infection, which is similar to HSV encephalitis, but limited and non-lethal. The early host response included microglial activation beginning on day 1 post infection and astrogliosis beginning on day 3 post infection. There was no detectable B or T-lymphocyte response or neutralizing antibody to HSV. Specific viral events included typical HSV pathological changes at infected sites, apoptosis and the presence of viral antigens. These studies suggest that the earliest response of the neonate to the presence of HSV in the brain consists primarily of constitutive elements. Adaptive responses were not detected during first seven days of infection and a humoral response was undetectable six weeks after infection. Studies such as these may assist in studying

various early aspects of pathogenesis, determining the mechanisms involving activation of the host response in a neonate and the long-term effects of CNS damage among survivors of neonatal HSV encephalitis.

Future Directions:

An animal model of a controlled limited HSV induced lesion in the neonatal mouse and characterizing the host response has innumerable applications. As a tool for understanding the various aspects of the infection and the factors responsible for the tissue destruction, it could be useful in improvement in treatment of herpes encephalitis and management in order to limit the long-term effects and disabilities. The neurologic impairment is a result of permanent destruction of the neuronal cells which cannot regenerate and restore themselves because they are highly specialized and differentiated cells which no longer undergo mitotic cell division. Is it possible to repair this damage by transplanting Neural Stem Cells (NSCs)? It is completely unknown at this time if exogenously transplanted NSCs can repair the damage caused by this infectious agent. The described mouse model of the disease can be a very useful tool in this investigation.

Transplantation of NSCs has shown promising results in compensation, cell replacement and repair of the damaged CNS tissue due to various etiologies. Some of the animal models investigated to date include those that recapitulate the neurological changes seen in Parkinson's disease, Huntington's disease, Gaucher's disease, Stroke and spinal cord injury. In animal models of Parkinson's disease and Huntington's disease, where neurons in the substantia nigra and striatum respectively are destroyed by injecting a neurotoxin, implantation of NSCs results in cellular engraftment (Bjorklund A et al., 2000). However transplantation of NSCs that have been pre-differentiated towards to

specific neuronal lineage or embryonic neural tissue exhibiting some neuronal commitment results in persistent neuronal engraftment, establishment of neuronal connectivity and functional compensation (Studer L et al., 1998). Transplantation of embryonic stem cell-derived motor neuronal cells, pretreated in vitro with growth factors recovery from paralysis and restoration of functional properties in rat model of spinal cord injury (Deshpande DM et al., 2006). These are just a few studies which describe the potential of NSCs in repairing and regenerating CNS tissue.

Since the neurologic sequelae in the survivors of neonatal herpes encephalitis is also due to CNS tissue damage and considering all these studies, hypothetically the transplanted NSCs have the potential to regenerate the CNS tissue damaged due HSV infection in the neonate as well. Using the neonatal mouse model in which the early events of host response are characterized, this hypothesis can be tested under the following specific goals:

1. Endogenous stem cell response: To determine if the endogenous NSCs in infected neonatal mice show any proliferative response to the herpes infection, and try to replenish the lost tissue.
2. Transplanted stem cell response :
  - (i) Engraftment of the transplanted neural stem cells in the neonatal brain after HSV infection

- (ii) If there is any engraftment, migration of the engrafted NSCs and differentiation of the NSCs in to the specific cell types and CNS integration.

Preliminary experiments and results:

In vivo Bromodeoxyuridine (BrdU) labeling and detection:

Neonatal mice infected with HSVgH- were intraperitoneally injected with 100 mg/kg body weight of BrdU, on day 1 post infection and again on day 5 and 8 post infection. The brains were extracted at 3 and 10 days post infection and analyzed for presence of BrdU positive cells. BrdU is an analog of thymidine and it is taken up by all the proliferating cells which incorporate BrdU in place of thymidine in the newly synthesized DNA in S-phase. The BrdU thus labeled in these cells was detected by immunohistochemistry using anti-BrdU antibody (Fig. 24) (BrdU In-Situ Detection Kit, BD Biosciences). Uninfected brains from neonatal mice treated with intraperitoneal BrdU were used as controls.

In the initial experiments BrdU positive cells (i.e., proliferating cells), were observed to be present in all regions of the brains in both infected as well as uninfected brains, but highly concentrated in the dentate gyrus and the sub-ventricular zones. At either 3 or 10 days post infection, there was no apparent difference in the infected or

uninfected brains as well as right or left half of the infected brains. These results show that there is an abundance of proliferating cells in the neonatal brain, which did not increase or decrease after infection. High concentration of these cells in the dentate gyrus and sub-ventricular zone suggests that many of the proliferating cells could be endogenous neural stem cells.

Endogenous NSCs also express early protein markers specific for the lineage of cells which they will be differentiating into (NeuN for Neurons; GFAP for astrocytes; MBP for Oligodendrocytes and F4/80 for microglia). They can be identified by immunohistological staining for the cell specific marker. Therefore co-localization of BrdU with these cell specific markers can establish their lineage.

The next set of experiments was designed to detect the BrdU and GFAP co-localizing cells by double immunofluorescence staining for BrdU and the anti-GFAP antibody. Infected and uninfected brains from neonatal mice were treated with BrdU as mentioned earlier and compared.

Figure 24 : In vivo BrdU labeling of the proliferating cells in neonatal mouse brain: Brain sections of neonatal mice treated with intraperitoneal BrdU. The BrdU positive cells (brown) are indicated by arrows. The sections are counterstained using hematoxylin. BrdU positive cells were mostly seen in pairs. **(a)** BrdU positive cells in stage of cell division. **(b)** A pair of BrdU positive cells.

Co-localization of BrdU and GFAP at 3 days post infection:

A double immunofluorescent staining was performed to detect BrdU and GFAP (Fig 25) in serial sections of brains of BrdU treated infected and uninfected mice at 3 days post infection. Five non-overlapping sections were used for counting cells positive for BrdU, GFAP as well as BrdU and GFAP. Statistical analysis using t-test was performed to compare means of the corresponding groups of infected as well as uninfected brains.

Comparing the infected brains with the uninfected brains, there was no statistically significant difference in terms of the cells positive for BrdU as well as BrdU and GFAP (Fig. 26). But the GFAP positive cells count showed statistically significant difference when corresponding halves of the infected and uninfected brains were compared. This confirms the earlier findings regarding the observed astrocytic response described in host response.

Figure 25: Co-localization of BrdU and GFAP in the neonatal mouse brain: Arrow marks indicate the positive cells: (a) GFAP positive cells detected using Streptavidin-Alexafluor 488 (Molecular probes) and visualized under green fluorescence filter. (b) BrdU positive cells detected using Streptavidin-Alexafluor 594 (Molecular probes) and visualized under red fluorescence filter. (c) merged image showing BrdU and GFAP positive cells (yellow)

Figure 26 : Quantification of the cells positive for BrdU, GFAP as well as BrdU and GFAP on right and left halves of the BrdU treated infected and uninfected mice brains extracted at 3 days post infection. The results are expressed as averages of the cell counts from 4 non-overlapping sections from 5 mice in each group. * as well as ** indicate the corresponding groups which showed statistically significant differences ($p < .05$)

The preliminary experiments to examine the endogenous NSC response were not conclusive, although they open a new Chapter in the investigation of neonatal herpes encephalitis. The experiments failed to determine conclusively any proliferative response of the endogenous NSCs after HSV infection. One indication from these experiments regarding the absence of any endogenous NSC response is that there was no significant difference in the BrdU positive cells at 3 days post infection when infected and uninfected brains were compared. A thorough investigation at this time point comparing the infected, uninfected, sham infected brains along with brains from mice not receiving any BrdU is required. Earlier and later time points should also be similarly investigated, to get a more complete answer. Another factor to be considered is the beginning of BrdU treatment, which could probably be done before the infection. But such a treatment can result in labeling of the virus infected cells as well.

There are many questions to be answered when it comes to the response of the endogenous NSCs after neonatal herpes encephalitis. Is there an attempt to replenish the lost tissue? Is there any endogenous neural stem cell response at all in the infected brains after herpes encephalitis? If there is, what would be the predominant cell type? What are the signals for such a response? Can the response be enhanced or altered favorably by any growth factor treatment? At the same time if no endogenous NSC response is seen, that calls for investigation of any signals responsible. After getting the complete answer, transplanted stem cell response should be investigated.

Transplanted NSC response:

After getting the complete answer, transplanted stem cell response should be investigated by a series of experiments to evaluate the response. These studies will encompass analysis of the efficiency of engraftment and determination of the fate of the engrafted NSCs in terms of migration and differentiation. For these studies, highly characterized neural stem cells of C17-2 cell line can be used. It is derived from a Balb/C X C57/Bl6 mouse and propagated in vitro by introduction of a conditionally mortalizing gene *v-myc* along with the neomycin phosphotransferase gene (*neo*) as a selectable marker (Snyder EY et al., 1992). These cells have been shown to be capable of efficient and robust engraftment and cellular proliferation through neuroaxis. These cells can be labeled in vitro using BrdU and traced in vivo by Immunoperoxidase staining using anti-BrdU antibodies. These cells also express the bacterial *lacZ* gene which can be alternatively used as a marker to follow them after transplantation.

(i) Graft timing:

The lesions induced due to the HSV infection and the host response can affect the engraftment and differentiation of the transplanted NSCs. So the first sets of experiments would be designed to determine the period when the HSV-1 lesioned brain is most receptive and conducive for NSC engraftment. For these experiments, neonatal mice with unilateral HSV-1 induced CNS lesions would be transplanted with NSCs into lateral

ventricles at 1, 3, 5 and 7 days post infection. This will provide us with an opportunity to study the process of engraftment in relation to HSV infection and resulting inflammatory process.

Engraftment will be evaluated at 1, 2, 4 and 8 weeks post transplantation by X-gal histochemistry (Price J et al., 1987), and β -gal immunohistochemistry (Lynch WP et al., 1999). Alternatively NSCs prelabelled with BrdU in vitro can be used to transplant and localized in vivo by BrdU immunohistochemistry. Sham-infected Balb/C mice will be used as controls.

(ii) Graft Localization:

Transplanted NSCs are capable of extensive migration in response to various signals. Cell death (Magavi SS et al., 2000) which is part of HSV-1 infection may be one such stimulus for signaling. This signaling may induce NSC migration to the site of damage, presumably following a signaling molecule gradient. To test whether engraftment and migratory signals are elicited by HSV-1 induced lesions, NSC will be transplanted into the lateral ventricle opposite to the side of induced lesions. Transplanted mice will be evaluated at 1, 2, 4 and 8 weeks for presence and distribution of transplanted NSCs and scored on their proximity to the site of HSV-1 induced lesions. NSC distribution will be compared to the sham infected mice.

(iii) NSC differentiation and integration:

The next set of experiments will be directed to determine the fate of transplanted NSCs after differentiation into neurons, astrocytes, oligodendrocytes or microglia. This will be achieved by double immunohistochemistry, using X-gal and/or BrdU immunohistochemistry for NSC localization and antibodies specific for surface markers for the above mentioned cells (Flax JD et al.1998; Lynch WP et al.1996). Neurons will be identified using antibodies specific for neurofilament, neuron specific tubulin (TuJ1) and MAP2. Astrocytes will be identified using antibody specific for GFAP, and oligodendrocytes by antibodies against CNPase or myelin basic protein (MBP). Microglia and macrophages will be identified using RCA-I or antibodies to F4/80.

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