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Innate and adaptive host response during the initial phase of herpes simplex virus encephalitis in the neonatal mouse

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To study early events of neonatal herpes simplex virus (HSV) encephalitis and its sequelae, the authors 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–β-galactosidase antibodies 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 in the neonate to HSV encephalitis. *Journal of NeuroVirology* (2006) **12,** 365–374.

Keywords: apoptosis; astrocytes; encephalitis; herpes simplex virus; microglia

Introduction

Neonatal encephalitis due to herpes simplex virus-1 (HSV-1) is a devastating disease and if untreated results in mortality in more than half of the cases and permanent neurologic sequelae in most of the survivors. Treatment with intravenous acyclovir has significantly reduced the mortality but long-term morbidity remains high. The majority of neonatal HSV infections are acquired at birth and result in three forms of the disease; (1) encephalitis, (2) disseminated multiple organ disease, and (3) skin eye mouth (SEM) disease (Whitley, 2001). Disseminated disease as well as SEM usually evolves into encephalitis (Whitley *et al*, 1980).

Early events in HSV-1 neonatal encephalitis, pathogenesis, and the host response are not completely understood. Studies are hampered by the devastating rapid destruction of the central nervous system (CNS) by HSV in the neonatal brain. In order to study this pathological event more closely, we employed a defective HSV-1 called HSVgH−, a Disabled Infective Single Cycle (DISC) virus that can only complete one cycle of replication (Forrester *et al*, 1992). This virus has the ability to infect and destroy a single

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cell, but cannot spread to adjacent cells because the progeny virus lacks the essential surface glycoprotein gH required for attachment to the next cell. Using this virus, to limit rapid destruction of the CNS, our studies show that lesions produced by intracerebral inoculation of HSVgH– are similar to those seen with wild-type HSV, but limited in its scope of destruction. Infection with precise amounts of this virus resulted in survival of 75% of the neonatal mice having detectable CNS lesions. Our studies further show that although the host's innate response, i.e., microglia and astrocytes, were activated, the adaptive responses, including T and B lymphocytes, were not recruited into the brain during the first 7 days of infection.

Results

HSVgH– replication in complementing and noncomplementing cells

Initial studies were designed to verify that HSVgH– undergoes limited replication in noncomplementing cells. A replication study was carried out in noncomplementing Vero cells and complementing F-6 cells. All viral titrations were done on F-6 cells. The results showed that no infectious HSVgH– was detected at 48 h post infection in noncomplementing Vero cells (Figure 1), indicating that the virus was not able to undergo continuing rounds of replication. However, in complementing F-6 cells, the amount of virus produced increased for 48 h before achieving a plateau, indicating that the virus underwent multiple rounds of replication. These results verify that the HSVgH– undergoes limited replication in noncomplementing Vero cells, which lack the gH-encoding gene.

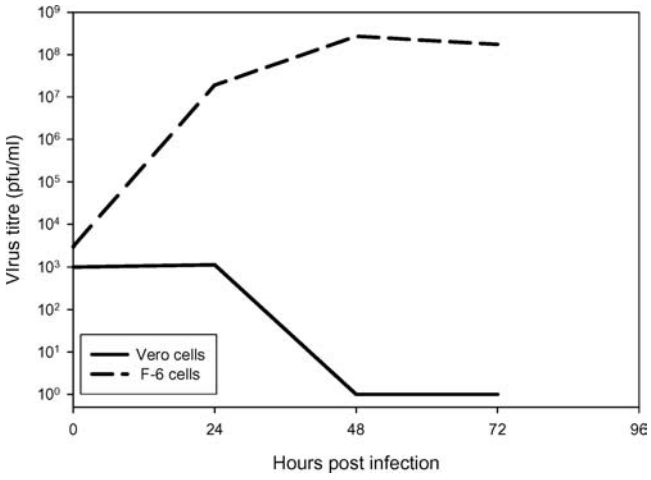

Figure 1 Replication of HSVgH– in noncomplementing Vero cells and complementing F-6 cells at 0, 24, 48, and 72 h post infection. F-6 and Vero cells were infected with HSVgH– at a moi of 0.01, frozen at the noted times, and plaque assays performed on F-6 cells.

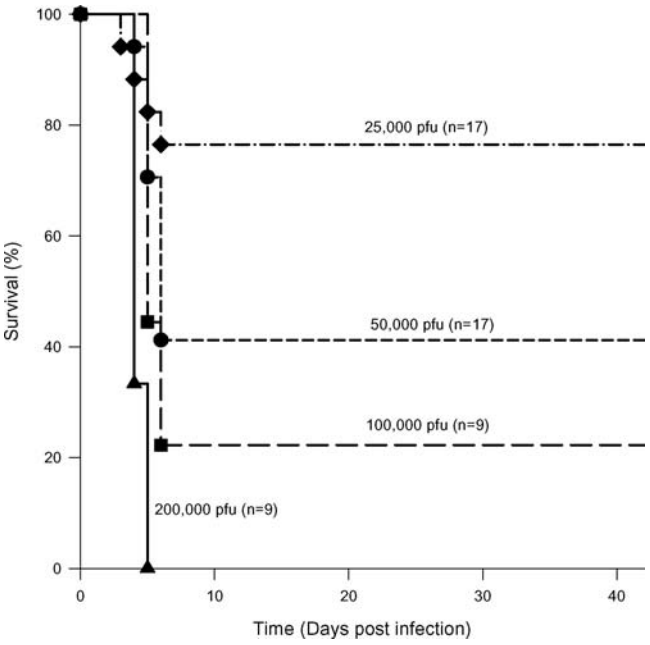

Figure 2 Kaplan-Meier survival analysis of 24-hour-old neonatal mice infected with 200,000, 100,000, 50,000 and 25,000 pfu of HSVgH– virus and observed for 6 weeks post infection. Experiments using 50,000 and 25,000 pfu of virus were carried out twice. (All four groups were compared using Gehan-Breslow test; $P < .001$).

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 2) 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 occured 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.

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

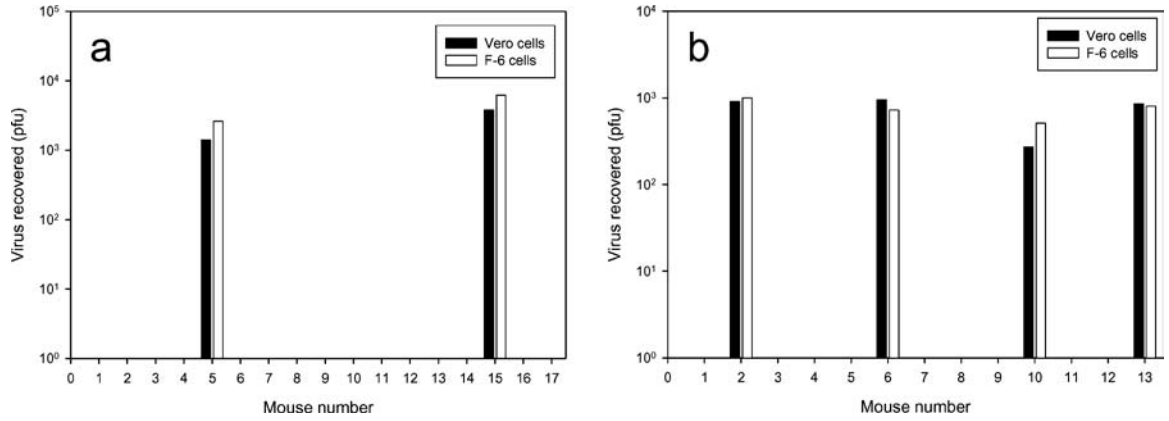

Figure 3 Replication competent viruses recovered by plaque assays using infected mice brain extracts. (**a**) At 1 day post infection a total of 17 mice were tested, mice 5 and 15 were positive. (**b**) At 3 days post infection 13 mice were tested, mice 2, 6, 10, and 13 were positive.

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. Therefore any plaques observed on Vero cells or F-6 cells would be due to virus with a wild-type phenotype. It follows that any recovered virus would have similar titers on both F-6 and Vero cells. 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 3a), 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 3b). 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 2) infected with the same amount of virus.

In vivo *demonstration of HSVgH– infection*

Brains were recovered from neonatal mice infected with 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 4a and b) 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 5a), 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 5b), 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.

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,

Table 1 Detection of infected and apoptotic cells

	Days post infection (positive/total)			
	1	*3*	*5*	*7*
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

Notes. 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.

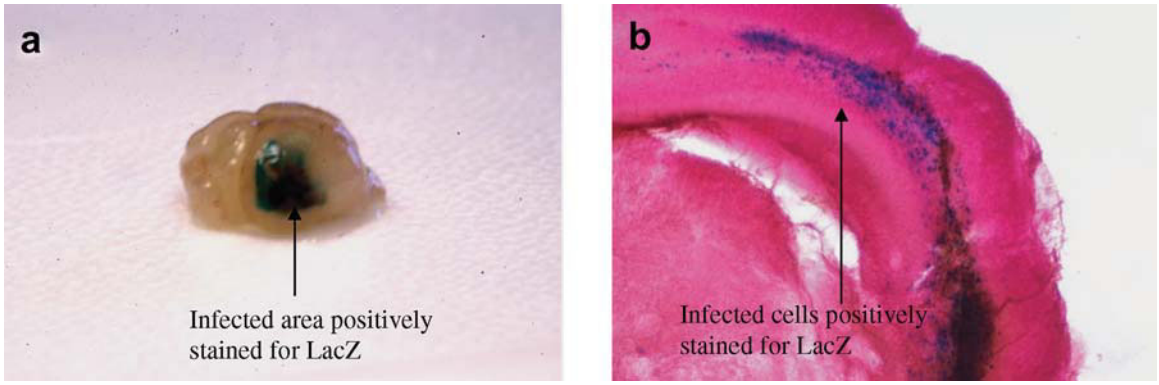

Figure 4 Brain extracted from a 24-hour-old Balb/C mouse infected with 25,000 pfu of HSVgH−, 1 day post infection and stained using X-gal histochemistry. (**a**) Whole brain stained with X-gal demonstrating the blue colored infected area. (**b**) Frozen section stained with X-gal (*blue*) and counterstained with neutral red.

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 6a, b and Table 1). At 5 and 7 days post infection, TUNEL-positive cells were not detected (Table 1).

Lack of neutralizing antibody in surviving mice

In order to determine if neutralizing antibodies formed in mice that survived infection with 25,000 pfu of HSVgH−, a plaque reduction assay was performed using sera from four mice, 6 weeks after they were intracerebrally injected with HSVgH−. The serum from each mouse was indepedendently tested

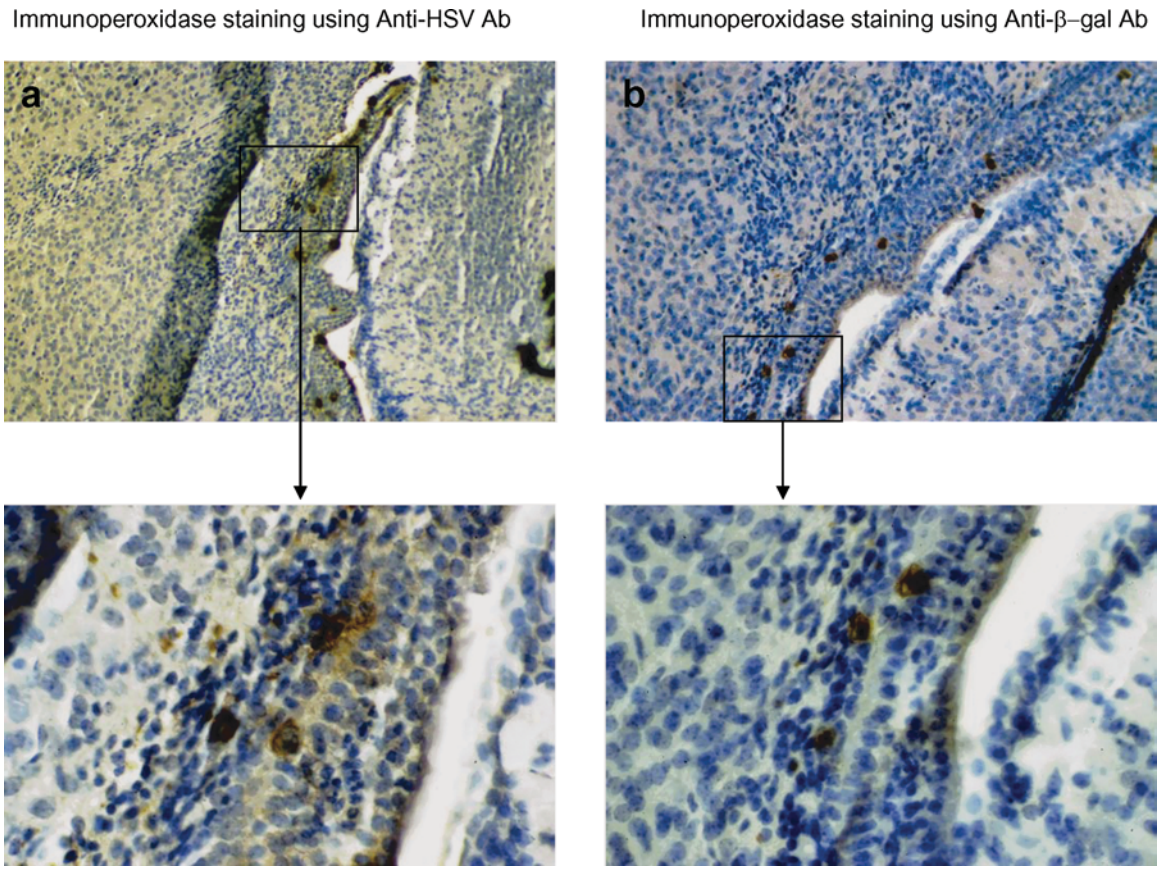

Figure 5 Immunoperoxidase-stained paraffin-embedded brain sections at 1 day post infection. (**a**) Section stained using anti-HSV antibody showing HSV-positive cells (*brown*). (**b**) Adjacent section stained using anti–β-galactosidase primary antibody showing β-galactosidase–positive cells (*brown*). Sections were counterstained using hematoxylin.

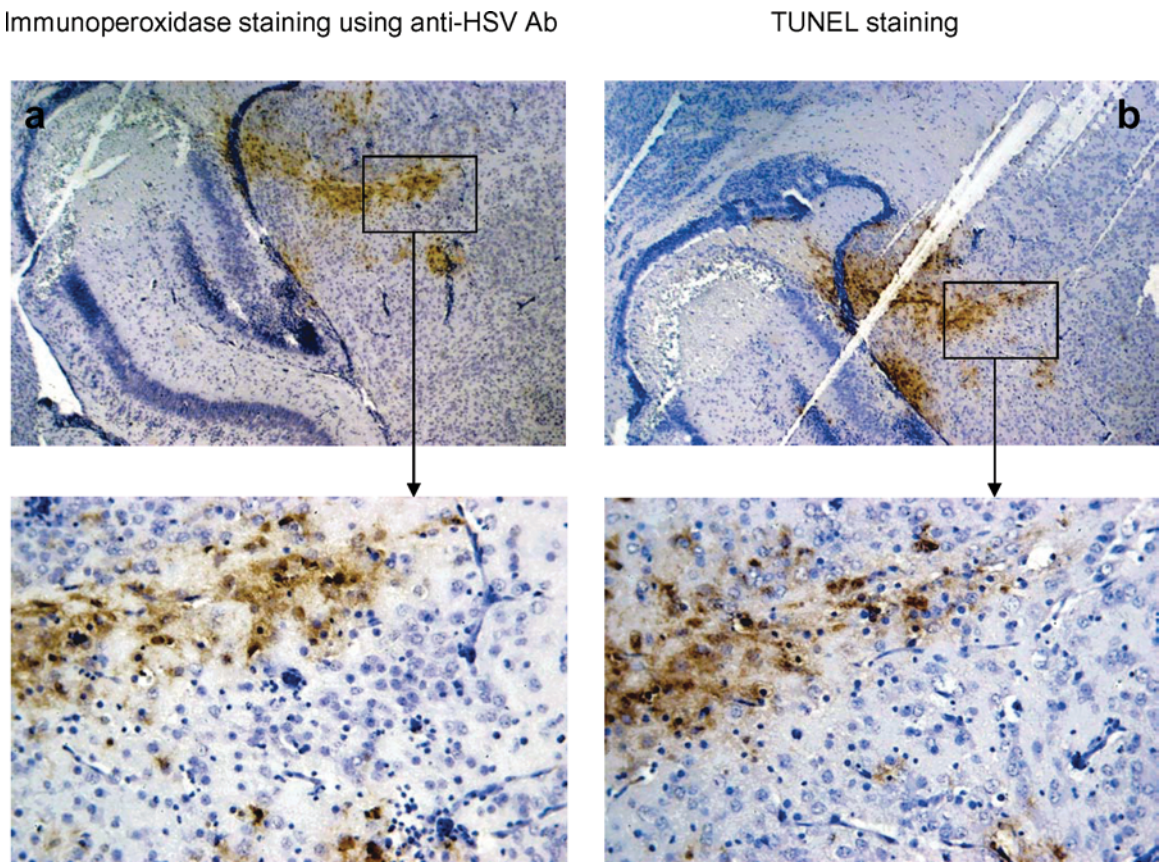

Figure 6 Adjacent brain sections at 3 days post infection. (**a**) Immunoperoxidase-stained section showing cells positive for HSV antigens (*brown*) and (**b**) TUNEL-stained section showing cells positive for apoptosis (*brown*) in the infected area shown in (**a**). The sections were counterstained using hematoxylin.

for neutralizing antibodies and the data were then combined. Results showed that there was no reduction in the number of plaques (data not shown), 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.

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 7a).

Table 2 Host response

	1dpi (positive/total)		*3dpi (positive/total)*		*5dpi (positive/total)*		*7dpi (positive/total)*	
Cell types (markers)	*Infected*	*Sham infected*	*Infected*	*Sham infected*	*Infected*	*Sham infected*	*Infected*	*Sham infected*
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

Notes. 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.

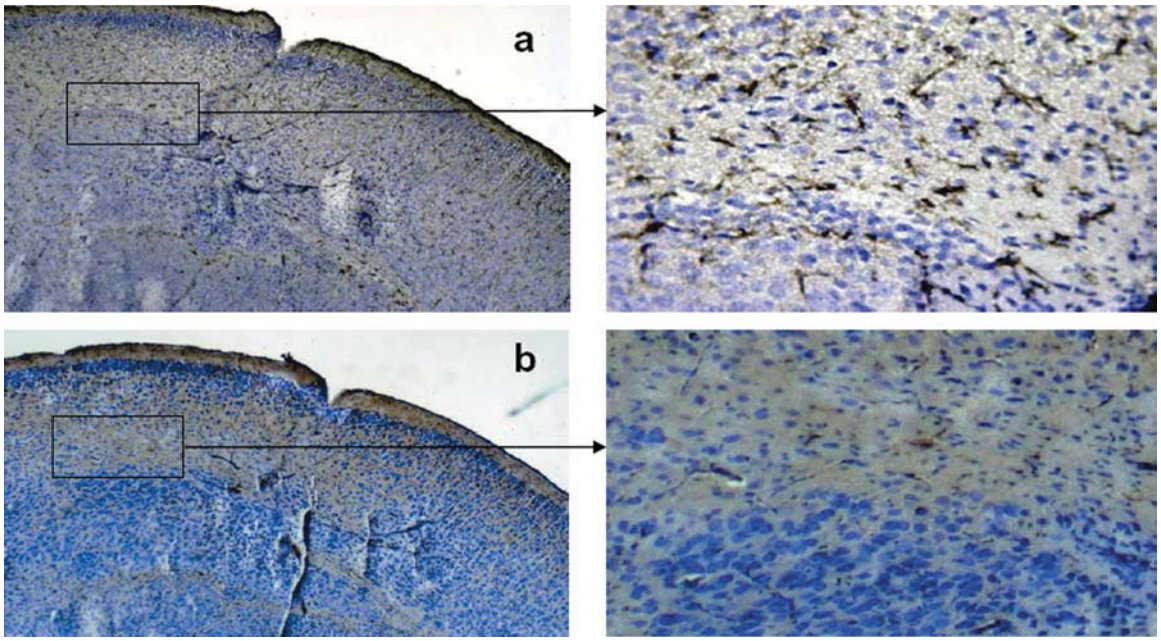

Figure 7 Microglial proliferation at 7 days post infection in the cerebral cortex of a mouse brain infected when it was 24 hours old with 25,000 pfu of HSVgH−. Paraffin sections were stained using RCA-I. (**a**) Infected mouse brain showing numerous positively stained microglia (*brown*) in the cortex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

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 7b).

Staining with anti-GFAP (Glial Fibrillary Acidic Protein) primary antibodies showed astrocytic proliferation in the cortex as well as white matter beginning primarily on day 3 post infection (Figure 8a) 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 8b).

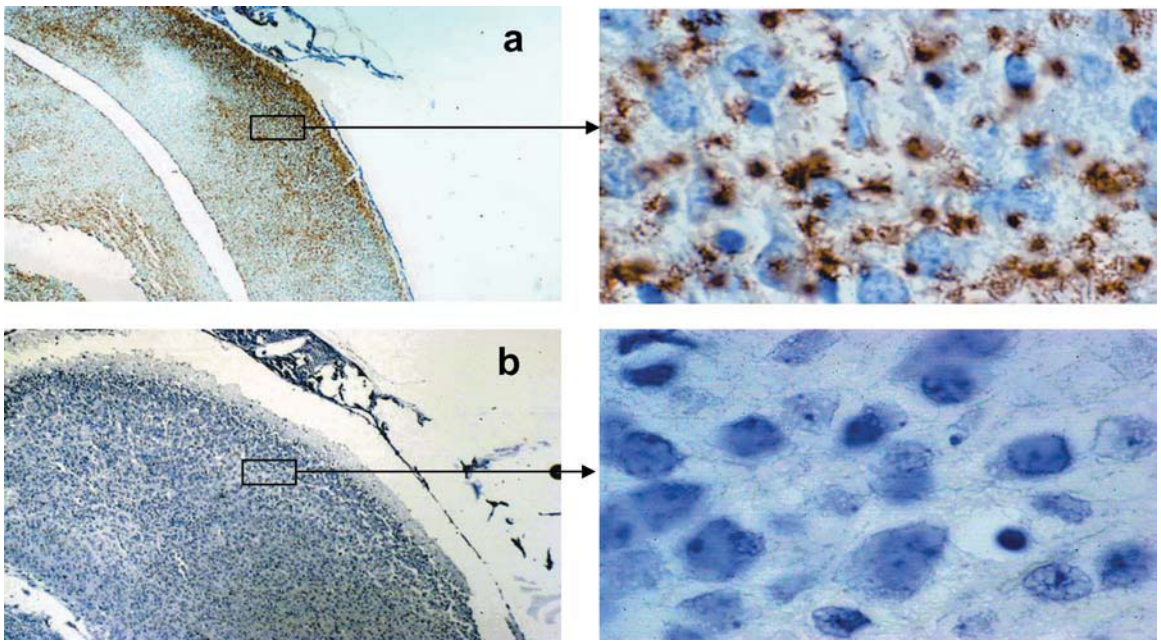

Figure 8 Astrocytosis at 3 days post infection in the cerebral cortex of a mouse brain infected when it was 24 hours old, with 25,000 pfu of HSVgH−. Paraffin sections were stained using anti-GFAP primary antibody. (**a**) Infected mouse brain showing numerous positively stained astrocytes (*brown*) in the cortex and (**b**) Sham-infected mouse brain showing no detectable astrocytes.

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

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, we utilized a virus that is incapable of continuous replication and generated an experimental infection in the neonatal mouse brain using an amount of virus 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 7 days of infection.

In a series of experiments we 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 because 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 *et al*, 2000). In addition, we were able to recover replication competent virus with wild-type phenotype 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 that 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 because LacZ expression in the infected cells is limited to early stages of infection because it is driven by the cytomegalovirus intermediate-early (IE)-1 promoter, which is likely 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 (data not shown).

Apoptosis is one of the responses to acute HSV infection that may aid the host by limiting replication and spread of the virus (Irie *et al*, 2004). Previous studies have described apoptotic cell death of infected cells and the bystanders in adult mouse brain (Shaw *et al*, 2002). Our results are consistent with these studies because we observed TUNEL-positive cells in sections adjacent to the HSV-positive sections at 3 days post infection. TUNEL-positive cells, however, were not detected on days 5 and 7 post infection. 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 noninfectious, they were unable to infect adjacent cells, leading to the presence of TUNEL-positive cells on days 5 and 7 post infection.

We examined the host cellular response to the presence of HSV in the neonatal brain. Astrogliosis characterized by GFAP immunoreactivity is a typical response seen in case of injury and inflammation to the adult CNS (Latov *et al*, 1979; Smith *et al*, 1983; Mathewson and Berry, 1985). The CNS injury may be due to mechanical trauma, neuronal necrosis, ionic changes (Mathewson and Berry, 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 and Dahl, 1974; Gearhart *et al*, 1979; Bernstein *et al*, 1981; Maxwell *et al*, 1990a and b). 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 *et al*, 1994). These investigators concluded that astrogliosis can occur in the neonatal brain if a sufficient stimulus is present (Balasingham *et al*, 1994) and inflammatory cytokines are elevated not only in adult animals but also in neonatal animals, associated with astrogliosis (Rostworowski *et al*, 1997). In agreement with these studies our results 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 nonspecific cellular response, we examined microglial response to the presence of HSV. Microglial activation is also a known result of cytokine release (Chan *et al*, 2001). Microglia and macrophages are thought to play an important role in the immune response in viral

encephalitis (Esiri and Kennedy, 1992). In our study 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.

We next examined the adaptive cellular response in the neonatal brain to HSV. Notably in our study, T and B lymphocytes were not detectable by immunoperoxidase staining using anti-CD3 and anti-CD20 antibodies during the first 7 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 and Loo, 1982). This latter observation is consistent with our results of a lack of T- and B-cell responses in the infected brains. However, we cannot exclude the possibility that in our system 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 our 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 our studies no lymphocytes were detectable, although neurons, microglia, and astrocytes themselves (Guilian, 1987; Logan *et al*, 1992; Wesselingh *et al*, 1990) 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 nonlethal. 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 7 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.

Materials and methods

Viruses and cells

SC16ΔgH (HSVgH−) virus 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 *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 *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). 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 Dubecco's modified Eagle medium (DMEM) medium (with 5% fetal bovine serum [FBS], 50 μg/ml gentamycin, and 2.25% sodium bicarbonate) and Vero cells were cultured using M-199 medium (with 5% FBS, 50 μg/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 noncomplementing cells

The replication of HSVgH− was examined in noncomplementing Vero and complementing F-6 cells. The cells were infected at a multiplicity of infection (moi) of .01 for 1 h and unattached virus was removed by washing the cells with medium. The infected cells were incubated at 37°C in a 5% CO_2 humid atmosphere. At 0, 24, 48, and 72 h 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 Committee. Balb/C mice obtained from Charles River Laboratories (Wilmington, MA) were bred and 24-hour-old pups used in all experiments.

Infection of neonatal mice
Neonatal mice were cryoanaesthetized in ice for 2 to 3 min 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.

Viral recovery from brain
Brains were recovered 24 and 72 h after infection and frozen at −80°C. The intact complete frozen brains were thawed and homogenized in 1 ml Tris-buffered saline (TBS) and centrifuged. 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 1 h. Controls included anti–HSV-1 antiserum, normal mouse serum, and TBS. At the end of 1 h, the samples were titered by plaque assay on Vero cells.

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

Microglia: Tissue sections were rehydrated, trypsinized for 10 min (Hauke and Korr, 1993), and incubated with biotinylated *Ricinus communis* agglutinin-I (RCA-I; Vector Labs) for 1 h. Sections were washed 3 times for 5 min each in phosphate-buffered saline (PBS) containing 0.1% Triton X-100. The sections were then incubated with streptavidin-HRP (horseradish peroxidase) (Jackson Immunoresearch) for 30 min, followed by a PBS rinse and a DAB (Diaminobenzidine) 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 h, followed by three washes of 5 min each in PBS and detection using DAKO EnVision+ system with DAB (Sigma Aldrich). 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 6 μm thickness using anti-HSV antibody (Biomeda) and 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
Brains infected with HSVgH− 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.

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