Major Histocompatibility Complex Antigens
in the Rat CNS
Following Herpes Simplex Virus Type 1 Infection

By

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ABSTRACT

The present study was designed to determine major histocompatibility complex (MHC) expression in the rat CNS following a viral infection. Male Wistar rats were anesthetized with ether and the right cornea was repeatedly scratched with a 23.5 gage needle. A 30 microliter drop of Herpes Simplex Virus Type 1 (HSV1) (PFU 33,000/mL) was placed on the scarified cornea. Animals were sacrificed 3, 6, 8, 10, 12, and 30 days following infection. Following decapitation, the brains were removed and placed in Zamboni fixative for immersion fixation. Monoclonal antibodies against rat MHC class I/class II antigens and HSV1 as well as polyclonal glial fibrillary acidic protein (GFAP) were used in both single and double staining procedures.

Neurons staining positively for HSV1 were observed in the ipsilateral principal sensory nucleus of the fifth nerve. Fifth nerve axons were also positive. Additional infected areas included midline brainstem structures, hypothalamus, thalamus, cerebellum and diffuse cortical regions. Infection became increasingly pronounced through day 10. Two animals who recovered from the viral encephalitis were sacrificed on days 12 and 30; they demonstrated much less positive HSV1 staining. In the infected animals, serial sections revealed both class I and class II positive staining of non-neuronal cells in brain areas which were also positive for HSV1. Endothelial cells and cells with microglia-like morphology were positively stained for class I and expression increased through day 10. The class II positive cells had the morphology of leukocytes and microglia-like cells with
increased expression occurring through days 8 and 10 throughout the brain. In serial sections, dense areas of class II positive microglia-like cells were located in the same areas as HSV1 positive clusters. MHC expression in the day 12 animal was quite pronounced with decreasing levels observed at 30 days. Tissue doubly stained for class II and GFAP demonstrated no overlap among positively stained cells suggesting that astrocytes were not expressing MHC glycoproteins. Astrocytes did, however, show morphological changes consistent with an acute CNS infection.

Microglia are an endogenous component of the CNS which may be phagocytotic, but the full range of functions which microglia possess is still unclear. The results of the present study indicate that MHC expression occurs in the CNS as early as 6 days following viral infection with increased expression through day 10. Continued high levels of class II expression at 12 days, following substantial active virus clearing, strongly suggest ongoing immune system activity which appears to be present at least as long as 30 days post-infection. Peripherally, MHC expression is involved in the induction of an immune response, suggesting that the rat brain is capable of mounting an immune response to viral infection. Microglia expressing class II antigens may possess the ability to present antigen to T cells and thereby initiate an immune response within the CNS. The present study suggests that MHC antigens may play an active role in viral clearance from the CNS.
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INTRODUCTION

Acute viral infections of the central nervous system (CNS) are responsible for a number of neurological disorders, such as encephalitis, meningitis, poliomyelitis, and demyelinating diseases (Johnson, 1982, Kristensson et al., 1983). Some viruses, such as the herpesviruses, have the capacity to produce latent infection with the possibility of reactivation and subsequent infection. Reactivation of latent virus has been implicated in various neurological and psychiatric disorders, such as Alzheimer's disease, schizophrenia, and multiple sclerosis (Kurstak et al., 1987, Fleming et al., 1983). Despite the severity of these infectious disorders, relatively little is known of the mechanisms of the immune response to viruses in the CNS.

Viral capsids and envelopes contain multiple virus-coded (antigenic) proteins which are T cell dependent (Johnson, 1982). The surface glycoproteins of a virus will activate T-helper cells which will in turn stimulate B cell antibody production. Production of neutralizing antibody to most viruses, including herpes simplex virus, is T-cell dependent. Phagocytosis and processing of both surface and internal virus-specific proteins will result in the synthesis of antibodies to all of these components. During infection, antibody can be directed against both circulating viral particles in the extracellular fluid or plasma and virus-coded proteins within the host-cell membrane. Circulating virions are inactivated or neutralized when antibody
attaches to them and forms an immune complex. However, not all circulating virus is neutralized and a small amount remains active. Antibody neutralization is clearly more effective in clearing extracellular virus than virus which travels intracellularly.

Most acute viral infections are associated with an initial inflammatory response characterized by an influx of polymorphonuclear and mononuclear cells (Johnson, 1982). The role of these cells in clearing virus from the CNS is poorly understood. The inflammatory response is followed by a specific immune response known as cell-mediated immunity (CMI). CMI involves specific T cell subsets as well as B cells and macrophages which work together to kill a virally infected host-cell. CMI is directed against either the virus-coded protein incorporated into the cytoplasmic membrane or a cell containing an integrated viral genome expressing cell surface virus-coded antigens. T-helper cell stimulated antibody production aids in host-cell destruction via antibody-dependent cellular cytotoxicity (ADCC). ADCC is mediated through killer and polymorphonuclear cells as well as macrophage phagocytosis.

Little is known about viral clearance from the CNS other than that it appears to be highly dependent on immune responses (Griffin et al., 1977). Virus appears to be phagocytosed by mesenchymally derived endogenous microglial cells (Johnson, 1982). That T cell responses may be more important than B cell responses in clearing virus is indicated by findings on natural immunodeficiency states in man. People with CMI defects may
develop a CNS infection to a virus which normally does not cause
disease (Johnson, 1982).

Recently, other factors involved in viral clearance from the
CNS have been uncovered. One important factor involves molecules
of the Major Histocompatibility Complex (MHC). MHC molecules are
known to play an important role in T cell-mediated immune
responses (Stites et al., 1987). The molecules which make up the
MHC consist of class I antigens (H-2 in rodents, HLA-A,B,C in
humans) and class II antigens (Ia in rodents, HLA-DR in humans).
Class I antigens are present on most nucleated cells while class
II antigens are largely restricted to immunocompetent cell types
such as macrophages, B-cells, and some T-cells (Stites et al.,
1987). These cell surface antigens play an important role in
self-recognition and antigen presentation during T cell-mediated
immune responses. MHC molecules are necessary for the initiation
of CMI. CMI involves cloning of specific T-helper cells, specific
T-cytotoxic cells, and specific antibody producing B cells which
function together to kill virally infected cells.

Although the importance of MHC antigens in immune reactivity
is generally accepted, a few studies have concluded that MHC
molecules are largely lacking in CNS tissue. In a recent review
article (Lampson et al., 1987), the distribution of class I and
class II immunohistochemical staining in both normal and
pathological tissue (animal and human) was described. Class I
staining was detected on blood vessel walls of normal animal and
human tissue. Neurons, glia, and other parenchymal cells were
negative. In similar tissue, rare class II positive cells and
vessel walls were seen, but most neural elements were negative. Pathological human tissue (largely physical trauma) showed no increase in class I expression. Class II reactivity was slightly increased in malignancies and following viral infection. The author concluded that MHC expression in both normal and pathological brain tissue was rare, suggesting little or no MHC activity in the CNS. In contrast to this conclusion, however, recent work in the area of MHC expression suggests an active role for MHC molecules in CNS immunity. Both in vitro and in vivo studies provide evidence in support of this hypothesis.

Model Systems

Under appropriate conditions, various cell types can be induced to express MHC antigens on their surface. Class I expression can be induced on all cell types in the CNS including neuroblastoma and ependymoblastoma lines following incubation with either gamma-interferon (IFN-γ) or interleukin-2 (IL-2) (DuBois et al., 1985, Lampson et al., 1983, 1984, Male et al., 1987, Suzumura et al., 1986, 1987, Ting et al., 1987, Wong et al., 1984, 1985). Class II expression has been demonstrated largely on cultured astrocytes and brain endothelium (DuBois et al., 1985, Fierz et al., 1985, Hirsch et al., 1983, Male et al., 1987, Wong et al., 1984). Cultured astrocytes will express class II antigens following incubation with IFN-γ (Barna et al., 1987, Male et al., 1987), activated T-cells (Fontana et al., 1984), or certain viruses (Massa et al., 1986, 1987). Brain endothelium will express Ia antigens following incubation with IFN-γ, phytohemagglutinin (PHA) or concanavalin A (conA) (Male et al.,
Furthermore, cultured astrocytes, macrophages, and microglia can be stimulated to produce interleukin-1 (IL-1), a lymphokine necessary for CMI, under appropriate conditions (Artursson et al., 1987, Giulian et al., 1986, Merrill, 1987).

**Normal Tissue**

**Animal** The highest concentration of class I expression in normal rat CNS tissue was found on vascular endothelial cells and on a few small glial cells (Akiyama et al., 1988, Matsumoto et al., 1987, Whelan et al., 1986). Class II expression was rare, but has been found on a small number of microglia, mononuclear cells, oligodendrocytes, and in choriod tissue (Akiyama et al., 1988, Matsumoto et al., 1986, Ting et al., 1981, Wang et al., 1987). In addition, T-helper lymphocytes (CD4+) have been found in small numbers in mouse, rat, and monkey brain (Hill et al., 1986, Neeley et al., 1987, Perry et al., 1987) with IL-1 binding noted in the rat (Farrar et al., 1987).

**Human** In normal human brain tissue, class I expression was largely confined to blood vessel walls and a few parenchymal cells. Neurons and glia were negative (Lampson et al., 1986, Sobel et al., 1988). Although class II expression was largely absent from normal tissue, a few HLA-DR positive astrocytes, microglia, and endothelial cells were found (De Tribolet et al., 1984, Hauser et al., 1983, Hayes et al., 1987, Hofman et al., 1986, Lampson et al., 1986, McGeer et al., 1987a, 1987b, 1988, Natali et al., 1981, Sobel et al., 1988). Small numbers of T-
helper cells have been found in the thalamus, cerebellum, and pons (Hill et al., 1986).

Pathological States

Animal models employing a variety of insults to the CNS have demonstrated enhanced MHC expression in brain tissue. MHC expression appears to follow a fairly predictable time course.

1) Experimental Allergic Encephalomyelitis (EAE) EAE is an immunologically-mediated demyelinating disorder often used in studying MHC expression. Currently, it is considered the best animal model for multiple sclerosis (MS). Spinal cord homogenate from allogenic rodents is injected subcutaneously into host rats producing reliable motor deficits as well as histological evidence of demyelination. The disease often exhibits relapsing and remitting phases. A large number of EAE studies have reported similar findings. Following the induction of EAE in rats, class I staining was found largely on dendritic and endothelial cells while class II staining was seen in cells questionably identified as dendritic cells, astrocytes (Matsumoto et al., 1986a, 1986b, Sakai et al., 1986), macrophages (Craggs et al., 1985, Sobel et al., 1984, 1987), microglia (Antoniou et al., 1987, Hickey et al., 1988, Sedgwick et al., 1987), and vascular endothelial cells (Craggs et al., 1985, Hickey et al., 1985, Sobel et al., 1984, 1987, Wilcox et al., 1987). In addition, T-cell subsets found predominantly in perivascular cuffs included T-helper (CD4+) and T-cytotoxic/suppressor (CD8+) cells (Ellerman et al., 1988, Merrill, 1987, Sedgwick et al., 1987, Sobel et al., 1987). B-cells also appeared to play a role in EAE (Willenberg et
In the preceding studies, immunohistochemical analysis of CNS tissue was performed using monoclonal antibodies against MHC antigens, T-cell subset determinants, microglia, monocyte/macrophages, and glial fibrillary acidic protein (GFAP) for astrocytes. Although class II expression on astrocytes was reported, most studies have found astrocytes to be negative for MHC glycoproteins.

The time course for the appearance of MHC antigens following the induction of EAE was as follows. MHC antigens appeared within the first week of the acute phase and peaked at approximately 10-18 days. Expression began to decrease by 1.5-2 months or during the remission phase. MHC expression was rare during remission (Antoniou et al., 1987, Craggs et al., 1985, Sakai et al., 1986, Sedgwick et al., 1987). The results from these studies demonstrate both enhanced MHC expression and lymphocyte infiltration during the acute phase of a demyelinating CNS disease, suggesting that MHC antigens play a significant role in antigen presentation and T-cell-mediated immunity in the rat brain.

2) Kainic Acid (KA) Lesions In a recent study, (Akiyama et al., 1988), epidural application of KA to the cerebral cortex was used to examine MHC expression. This procedure causes considerable neuronal damage to the underlying tissue, resulting in focal areas of enhanced MHC expression. Immunohistochemical analysis was carried out with monoclonal antibodies against rat MHC antigens and leukocyte common antigens (LCA). Polyclonal antibody against GFAP was used to detect astrocytes. Cells
staining positively for class I included round cells (possibly macrophages) and microglia. Round cells, "ameboid" cells, and microglia were positive for class II, with both round cells and microglia also positive for LCA. Neurons did not express detectable levels of class I, class II, or LCA at any time. Double immunohistochemical staining revealed most LCA positive cells to be class I positive with only a few cells doubly stained for both LCA and class II. In addition, most class II cells stained for class I, but the reverse was not true. Astrocytes (GFAP+) were negative for both class I and II.

The time course for MHC expression in KA lesioning was similar to that seen in EAE. MHC antigens appeared within the first week following KA application, peaked shortly afterwards, and declined over the following 16-20 weeks. Class II expression declined somewhat more rapidly than class I expression.

As in the EAE studies, these results suggest MHC involvement in the rat CNS, as well as a role for microglia in antigen presentation. Astrocytes (GFAP+) did not doubly stain for class II.

3) Transplant/Graft vs. Host  CNS tissue transplants, unlike peripheral transplants, do not appear to result in total tissue rejection (Medawar, 1948). This is often used as evidence for immunological privilege in the CNS. However, a few studies have demonstrated enhanced MHC expression, as well as lymphocytic infiltration, following transplantation of brain tissue into the CNS. In one study, detectable levels of MHC expression were seen in the brain following transplantation of foetal neocortical
tissue into the lateral ventricle of allogenic mice (Nicholas et al., 1987). Class II positive endothelial and parenchymal cells, both major T-cell subsets and macrophages were found. Hickey and Kimura (1987), demonstrated a graft versus host immune response in the rat CNS which resulted in the appearance of class I positive vascular endothelial cells as well as class II positive endothelial, parenchymal, and microglia cells. In another experiment, brainstem pieces grafted into the cerebellum of allogenic mice resulted in the appearance of both T-helper and T-cytotoxic/suppressor lymphocytes (Date et al., 1988). The above studies employed immunohistochemical techniques using monoclonal antibodies against mouse MHC antigens, T-cell subset determinants, and macrophages.

Although CNS tissue transplants do not result in a vigorous immune response, the results of these transplant studies suggest that some immune reactivity may still occur. The reasons behind failure of a robust immune response and transplant rejection following tissue transplant in the CNS currently remain unclear.

Human MHC expression in the CNS has been reported in a few studies examining human pathological tissue. A number of different cell types have been reported to express MHC antigens in multiple sclerosis (MS) plaques. Class I positive astrocytes have been found in active MS lesions (Traugott, 1987), as well as class II positive microglia (Hayes et al., 1987, Woodroofe et al., 1986), macrophages (Esiri, 1987, Traugott et al., 1984), astrocytes (Hofman et al., 1986, Traugott et al., 1985, 1987), and endothelial cells (Traugott et al., 1985). Post-mortem brain
tissue from patients diagnosed with Parkinson’s or Alzheimer’s disease revealed class I and II positive microglia as well as T-cells. (Itagaki et al., 1987, McGeer et al., 1987a, 1987b, 1988, Rogers et al., 1988). Astrocytes did not show class II staining. Tumor and glioma brain tissue was examined for MHC expression. Vessels stained positively for class I while class II reactivity was visualized on astrocytes, macrophages, and a few microglia-like cells (Bhondeley et al., 1987, Franks et al., 1986, Lampson et al., 1986, Rossi et al., 1987). Although additional pathologies such as viral infection (Abbott et al., 1987, Boutin et al., 1987, Goudsmit et al., 1987, Manuelidis et al., 1987, Petito et al., 1986, Richert et al., 1987, Zeman, 1978), systemic lupus erythematosus (Ernerudh et al., 1984), and schizophrenia (Ahokas, 1986, Roberts et al., 1987, Stevens, 1982) have been examined for MHC expression, no conclusive evidence has been reported at this time.

**Purpose of the Present Study**

Viral infections have only recently been utilized to examine MHC expression in the CNS. A research team in Sweden reported a marked increase in both class I and class II antigen expression (immunohistochemical analysis) in the rat brain following the induction of measles virus encephalitis (Olsson et al., 1988). Although specific cell types were not described, distribution of the MHC molecules was more widespread than the occurrence of viral antigen, suggesting a soluble factor for their induction. In surviving animals, T-cell subsets were also noted perivascularly and in the brain parenchyma.
The present study was designed to determine MHC expression in the rat CNS following corneal inoculation with herpes simplex virus type 1 (HSV-1). This is the first report to examine MHC expression and delineate a detailed time course of this antigen expression in the rat CNS following HSV-1 infection.

HSV1 has been used to produce CNS viral infection in animals via multiple routes of inoculation. MHC expression, however, has not been clearly documented. Two studies described an inflammatory cell infiltration in the CNS region of the trigeminal nerve root entry zone (TREZ) following either peripheral or snout inoculation with HSV-1 (Kastrukoff et al., 1987; Kristensson et al., 1983). The cellular infiltrate consisted of lymphocytes, macrophages, and a few plasma cells. In other laboratories, intracerebral, intravenous, and sciatic nerve inoculation as well as hippocampal microinjection led to lymphocytic meningitis with perivascular lymphocytic cuffing (Anderson et al., 1983, McFarland et al., 1986, 1987). Following corneal HSV-1 inoculation, both T-cells and a few scattered B-cells were found in the CNS region of the TREZ (Townsend, 1985). In one additional study, rabbits inoculated intrathecally or via corneal scarification developed CSF mononuclear pleocytosis during the acute phase of infection, followed by elevated CSF immunoglobulin levels (IgG, IgA) during the recovery phase (Laskin et al., 1987). Behaviorally, the acute encephalitis was characterized by hypoactivity, diminished food and water intake, and hunched posture, most often followed by death. Although little work has been done to date on MHC expression in the CNS
following an HSV1 viral infection, it is an area which should provide a considerable contribution to the understanding of CNS immunity.

Herpes simplex is frequently associated with human CNS disease. Initially the cause of a mild disorder such as cold sores, HSV infection may become more serious when the CNS is invaded. HSV1 is the cause of a majority of cases of sporadically occurring acute necrotizing encephalitis in the Western world with an estimated 4,000 cases yearly in the United States (Viken et al., 1978). Although other viruses more commonly produce an encephalitic illness, morbidity and mortality resulting from herpes simplex encephalitis exceeds that of other infections. The mechanism responsible for development of the encephalitis remains poorly understood.

Clinically, an individual suffering from herpes encephalitis presents with headaches and seizures. These symptoms are frequently associated with psychiatric disturbances and selective memory impairment (Viken et al., 1978). The clinical course is characterized by rapid changes over a period of a few days, resulting in stupor which often progresses to coma. Most cases of HSV encephalitis result in either death, or resolution with variable degrees of intellectual, memory, or emotional deficits. Few individuals recover fully.

Pathologically, lesions are usually limited to the subfrontal and medial temporal regions of the brain (Viken et al, 1978). Hemorrhagic lesions and congestion of small vessels are often grossly visible. The infection produces a necrotizing
process in which neurons, oligodendrocytes, and astrocytes are affected. Inclusions have been noted in all three cell types, but infection of endothelial cells has not been identified. Post-mortem tissue from surviving individuals reveals cystic necrosis of the brain.

Latent virus persists in the natural host in a non-infectious form and may remain latent for years (Johnson, 1982). The precise nature and intracellular site of the latent stage are unknown, but the virus is thought to be sequestered in neurons of sensory ganglia (Stevens, 1978). Reactivation may follow febrile illness, stress, X-ray irradiation, or a variety of other non-specific events, but the mechanisms involved in reactivation are poorly understood. Since reactivation can occur in the presence of antibody, cell-mediated immune responses have been implicated (Stevens, 1978). Reactivation of latent virus in aged individuals with a compromised immune system has been hypothesized as implicated in the etiology of certain degenerative CNS disorders, such as Parkinson’s and Alzheimer’s disease (Kurstak et al., 1987, Rytel, 1987).

Pathogenesis of HSV1 Infections of the CNS

HSV adsorption to astrocytes and synaptosomes is thought to occur through HSV type-specific receptors (Johnson, 1982, Lycke et al., 1984, Vahlne et al., 1978). Receptor mediated attachment is probably essential for neuritic uptake of virus which is then followed by fusion of the viral envelope with the host-cell membrane. A complex series of events follows, resulting in the replication of the virus and the establishment of latency
Once the virus enters the CNS, lysis of infected cells appears to be important in allowing enveloped virus to enter the extracellular space where adsorption to glial cells can occur (Townsend, 1985). HSV has been shown to travel transynaptically in both a retrograde and anterograde direction (Fleming et al., 1983, Kurstak et al., 1987, Lycke et al., 1984, Ugolini et al., 1989, Vahlne et al., 1978, Viken et al., 1978). It has been suggested that HSV may serve as a tracer because replication of virus in the recipient neuron following transfer amplifies the "tracer signal" (Ugolini et al., 1989). However, due to the concurrent spread of virus within glial cells, this technique is limited.

It is thought that both the centripetal and centrifugal neural transmission of HSV may be important in the induction of latency (Viken et al., 1978). Little is understood about the process of latent infection other than it is likely established in neurons of sensory ganglia where viral information is conserved without necessitating production of infectious virus (Fleming et al., 1983, Kurstak et al., 1987, Viken et al., 1978).

HSV is apparently capable of infecting the full range of CNS cell types in a number of different species (Viken et al., 1978). The productive infection is characterized by an alteration in the nucleus consisting of dispersion of chromatin to the nuclear margin and development of eosinophilic inclusions within the nucleus. Some infected cells may not show histologically apparent
changes while other cells may undergo degeneration even though they may not be directly infected by the virus.

Corneal inoculation produces a relatively slow and reliable CNS infection (Fleming et al., 1983, Tomlinson et al., 1983, Townsend, 1985). Intracerebral inoculation, which is commonly used to model human HSV infection, usually results in rapid death (1-6 days) (McFarland et al., 1986). Infection initiated in the cornea allows the spread of virus to occur relatively more slowly, therefore providing an increase in the time span of infection (1-2 weeks). In addition, more animals are likely to recover from the encephalitis following corneal inoculation.

The cornea is innervated by the ophthalmic branch of the trigeminal nerve and a number of studies have demonstrated a replicable infection in the trigeminal nerve following corneal inoculation (Fleming et al., 1983, Tomlinson et al., 1983, Townsend, 1985). Other infected areas reported were the trigeminal ganglion, thalamus, hypothalamus, and cerebellum. Viral particles were found in neurons, oligodendrocytes, and astrocytes. Corneal inoculation with HSV1 apparently results in sequestration of virus by the trigeminal nerve, which transports the virus back to the CNS. Once in the CNS, infection continues to spread, thus establishing a reliable viral infection.

Predictions

HSV1 will be employed to establish an acute CNS infection in the rat via corneal scarification. Based on the information gleaned from the literature, changes in the following dependent variables are likely to ensue.
1) Using antibodies against HSV1, we are likely to see infected neurons initially in the trigeminal nucleus. Glial cells are also likely to be infected and the infection may spread with time.

2) Using antibodies against MHC class I and II antigens, cells in various brain regions will probably stain for these antigens and it is probable that the cell type(s) expressing these antigens will be non-neuronal.

3) The MHC immunoreactivity is likely to be most intense in areas staining for HSV1.

4) Brain sections doubly stained for MHC class I or II and glial fibrillary acidic protein (GFAP) will likely aid in identification of MHC immunoreactive cells.

5) Examining a number of different post-infection time periods will likely contribute to a detailed map of HSV1 spread in the CNS as well as topography of MHC expression.

If MHC immunoreactivity is found in the CNS, we may infer the following. MHC molecules are likely to be integrally involved in the process of viral clearance from the CNS. Furthermore, the cell types expressing these antigens are likely to play an essential role in the immune response. This is important because so little is known about viral infections in the CNS and the processes involved in the immune response following viral infection. It is therefore essential to examine closely MHC expression in the CNS following HSV1 infection to gain a better understanding of its role in CNS immunity. Such understanding may point to improved therapeutic intervention.
MATERIALS AND METHODS

**Virus**: Herpes simplex virus type 1 (strain F) was obtained from the American Type Culture Collection (Rockville, MD) ATCC VR-733. Virus stocks were grown in human foreskin diploid fibroblast (HDF) cells. The cells were grown in Dulbecco's modified Eagles Medium (DMEM) with 10% foetal bovine serum (FBS) and 100 units/ml of Penicillin and Streptomycin. The virus was initially purified by plaque purification on HDF cells and one plaque was picked and amplified in these cells. The final stocks were prepared by infecting 20 x 100 mm petri dishes of HDF cells with virus at a multiplicity of infection of 0.001. The virus was harvested 24 hours after the cells showed 100% cytopathic effect (4 days post-infection). The supernatant was harvested into centrifuge bottles and the cell debris removed by centrifugation at 5000 rpm for 10 min at 40°C. The supernatant was frozen in aliquots of 1 ml and stored at -70°C. The virus was titered in HDF cells utilizing plaque assay procedures. Serial dilutions of virus were applied to duplicate 60 mm petri dishes of HDF cells. The virus was absorbed for 1 hour. The liquid was then removed and replaced by an overlay of DMEM with 5% FBS and 0.5% agarose. Plaques were visible 2-3 days later (5.7 x 10 pfu/ml). The final plaque assay of the stock was performed on Vero cells (3.3 x 10 pfu/ml).

**Virus Application**: Male Wistar rats (250-300 gm) were anesthetized with ether and the right cornea was repeatedly scratched with a sterile 23.5 gage needle. A 30 µl drop of Herpes Simplex Virus Type 1 (PFU 33,000/ml) was placed on the scarified
cornea and animals were returned to their cages with ad lib food and water until time of sacrifice. Control animals received corneal scratching without application of virus. Rats were sacrificed after periods of 3, 6, 8, 10, 12, and 30 days. Two to four rats were used for each of the 3, 6, 8, and 10 day time periods and control animals were sacrificed on days 6 and 10. Days 12 and 30 had a single rat for each time period.

**Tissue Preparation:** Under deep sodium pentobarbital anesthesia, rats were decapitated and the brain, trigeminal ganglia with the proximal nerve roots, spleen, thymus, and liver were immediately removed. The brains were cut into 5 mm coronal sections and all tissue was fixed in a solution of 2% paraformaldehyde and 1% picric acid in 0.1 M phosphate buffer (pH 7.4) for 48 hours. Tissue was transferred to phosphate-buffered 15% sucrose solution for at least 2 days, and then sectioned on a freezing microtome at a thickness of 30 μm. Sections were collected in phosphate-buffered saline containing 0.3% Triton X-100 and stored as free-floating sections at 4°C until stained.

**Immunohistochemistry:** Monoclonal antibodies against rat MHC antigens were obtained from Sera-lab (England) as supernatants of culture medium. OX6 was used against a monomorphic determinant of MHC class II (Ia) antigen (McMaster et al., 1979) and OX18 was used against a monomorphic determinant of MHC class I antigen (Fukumoto et al., 1982). Dilutions were 1:100 for OX6 and 1:300 for OX18. Polyclonal antibodies against HSV1 (Dako, Denmark) and rabbit anti-glial fibrillar acidic protein (GFAP) (Dako,
Denmark) were used at dilutions of 1:1000 and 1:10,000 respectively. GFAP was used to identify astrocytes.

Immunostaining was carried out using the ABC method (Hsu et al., 1981) with a few modifications (Kaneko et al., 1987). Following 30 minutes pre-treatment with 0.2% hydrogen peroxide in PBS and 3 buffer washes (x20 min), sections were incubated with primary antibody for 24 hours at room temperature. Sections were then incubated with biotinylated anti-mouse IgG (H&L) or anti-rabbit IgG (Vector, 3.75 µg/ml) solution containing 2% normal rat serum for 2 hours at room temperature. This step was followed by incubation with ABC solution (Vector) diluted 1:400 for 1 hour at room temperature. The peroxidase reaction was carried out with a solution of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.01% hydrogen peroxide and 25 mM nickel ammonium sulfate in 0.05M Tris-HCL buffer pH 7.6. The reaction product was purple in color with little or no background staining. Some sections were counterstained with neutral red and all sections were mounted on glass slides and cover-slipped with Entellan.

MHC class II and GFAP antibodies were used in a double immunostaining procedure. Double immunohistochemical staining was performed in two staining cycles. During the first cycle (GFAP), the standard peroxidase-anti-peroxidase (PAP) method of Sternberger (Sternberger, 1979) was used. Sections were incubated with GFAP as the primary antibody followed by goat anti-rabbit IgG (Cappel, USA) at a dilution of 1:1000 overnight at 4°C. This step was followed by incubation with rabbit PAP (Dako, Denmark) for 2 hours at room temperature at a dilution of 1:1000. The DAB
reaction was carried out minus the nickel ammonium sulfate to produce a brown reaction product. Following a 30 minute treatment with 0.5% hydrogen peroxide, OX6 antibody staining was performed as describe for single immunostaining.

Peripheral tissue sections from the thymus, spleen, and liver were stained with antibody against HSV1 as described for brain sections and control staining sections were treated as described above with omission of the primary antibody from the procedure. Serial sections were also stained with cresyl violet.
RESULTS

Behavioral Changes

Animals inoculated with virus appeared healthy for the first 6 to 7 days and rapidly became moribund during days 7 through 10. The severely moribund animals (day 10) were still alive prior to sacrifice. All animals exhibited clinical signs characteristic of an encephalitis including hypoactivity, failure to groom, failure to consume food and water, and hunched immobility. Two animals recovered and appeared healthy, regaining activity and food consumption, when sacrificed at 12 and 30 days post-infection. Herpes Simplex Virus Type 1 (HSV1) Staining (Table 1)

At 3 days, a small amount of positive HSV1 staining was present in the axons of the fifth nerve as well as in a few scattered cells in the trigeminal nucleus. Viral infection was minimal at this stage. Days 6 through 10 were characterized by a significantly increased number of HSV1 positively stained cells (Figs.1,2). Systemic infection was ruled out based on negative staining of peripheral organs. HSV1 immunoreactivity was found initially in trigeminal nerve axons, brainstem nuclei and in the cerebellum. By the eighth and tenth days, positive staining was found throughout much of the brain. Infected areas included the hypothalamus, thalamus, and many diffuse cortical regions.
Table 1  HSV1 immunoreactivity within various brain regions over the course of 30 days.

<table>
<thead>
<tr>
<th>Region</th>
<th>Days Post-Inoculation</th>
<th>3</th>
<th>6</th>
<th>8</th>
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<tr>
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<td>III Nu (Edinger-Westphal)</td>
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<td>Caudate/ Putamen</td>
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<td>Cerebral Cortex</td>
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- = no visible staining
± = very little staining
+ = moderate staining (each additional + indicating increase in number of positively labeled cells)
R = right side (ipsilateral to inoculation)
L = left side
Fig. 1. Section through the brainstem of an animal 6 days after corneal inoculation. HSV1 immunoreactive cells within the spinal trigeminal nucleus (large arrow). Some HSV1 reactivity is also present in the suprafacial nucleus of the seventh nerve (arrowhead). VII n., facial nucleus. (Bregma (B)=−11.3 mm, according to Paxinos and Watson, 1982) Bar=500 μm.
Fig. 2. Section through the brainstem of an animal 8 days after inoculation HSV1 immunoreactive cells increasing in number from day 6. V n., spinal trigeminal nucleus; VII n., facial nucleus; IV, fourth ventricle; m, midline. (B=-11.3 mm) Bar=500 μm.
At 12 days, very few positively stained neurons remained and only rarely were HSV1 immunoreactive neurons noted at 30 days. HSV1 reactivity was initially ipsilateral at 6 days, and became increasingly bilateral through day 10. Distribution at 12 days was ipsilateral.

Day 6

At this stage, the infection was largely confined to the trigeminal nerve and cerebellar cortex. Trigeminal axons and large neurons within the spinal trigeminal nucleus were positively stained for HSV1 (Figs.3,4). Neuronal somas were approximately 20 μm in diameter, with visible dendritic processes, characteristic of a typical neuronal profile. Smaller, round cells with an approximate diameter of 5-10 μm and no visible processes, were also positively stained, but were difficult to identify from the stain (Fig.5). Given the characteristics of these cells, they were most likely leukocytes. A small focal lesion was present in the Edinger-Westphal nucleus of the third cranial nerve. A few HSV1 positive neurons in the granular layer of the cerebellum were noted. Morphologically, these neurons were approximately 25 μm in diameter with long processes and dendritic branches extending throughout adjacent cerebellar layers (Fig.6). Based on morphology and location of these neurons, they are most likely Golgi neurons.
Fig. 3. Section through the trigeminal nerve of an animal 6 days after inoculation. HSV1 positively stained axons on the ipsilateral side, demonstrating axonally transported virus. Bar=50 μm.
Fig. 4. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. HSV1 positive neuron with fine processes. Note beaded appearance of processes in neuropil. Neighbouring negatively stained neurons (possibly non-infected) indicate similarity in size to infected cells. (B=-11.3 mm) Bar=50 μm. (See A-A’ in Fig. 25).
Fig. 5. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. HSV1 reactive small, round cells, approximately 5-10 μm in diameter with no visible processes. These cells are most likely leukocytes. (B=-11.3 mm) Bar=50 μm.
Fig. 6. Section through the cerebellar cortex of an animal 6 days after inoculation. Large HSV1 reactive neurons approximately 25 μm in diameter are present within the granular cell layer. Note the fine dendritic branches extending into adjacent layers. As described by Eccles et al. (1967), Golgi neurons are located within the granular layer of the cerebellar cortex and possess dendritic branches which often extend into adjacent layers. gr, granular layer; mo, molecular layer. (B=-10.3 mm) Bar=50 μm.
Day 8

By 8 days, the infection had spread to a large number of brain regions. The number of HSV1 positive neurons within the trigeminal nucleus had increased substantially. Layers 1-3 of the cerebellar cortex and round cells within the inferior cerebellar peduncle (ICP) were also infected. The cells within the ICP were small, round, and approximately 5-10 μm in diameter with no visible processes. Similar to the cells noted in the trigeminal nerve, these cells were most likely leukocytes. Positively stained cells in the cerebellum were largely confined to the granular layer and were also small and round in shape.

At this stage, both the thalamus and the hypothalamus were infected. Substantial infection in the thalamus was present mainly in the ventral posterolateral (VPL), ventral posteromedial (VPM), and lateral geniculate nuclei (LGN). Large cells within the VPL and VPM were positively stained and a large focus of infection within the ventroposterior thalamic nucleus (parvocellular region) was also HSV1 immunoreactive (Fig.7). Within the hypothalamus, the posterior, dorsal, and lateral hypothalamic areas were infected. Many large neurons were positively stained (Fig.8). A small number of lightly stained cells with a tufted appearance were evident within the anterior olfactory nucleus.
Fig. 7. Section through the ventroposterior nucleus (parvocellular region) of the thalamus of an animal 8 days after inoculation. A large focus consisting of HSV1 immunoreactive neurons, small round cells, and neuropil is located lateral to the third ventricle. (B=-3.8 mm) Bar=100 μm. (See B-B' in Fig.25).
Fig. 8. Section through the paraventricular hypothalamic nucleus of an animal 8 days after inoculation. Note HSV1 reactive neurons with fine dendritic processes. (B=-1.8 mm) Bar=50 μm.
Small focal areas of positive staining were present in a number of different brain regions. Neurons and smaller, round cells in the caudate/putamen and lateral septal nuclei were positive for HSV1 and the number of positive cells within the oculomotor nucleus had increased. The frontoparietal cortex (Brodman’s areas 3,2,1) (Krieg, 1946) was also infected. Cells with pyramidal or fusiform morphology were positive for HSV1 in the internal and external pyramidal layers of the frontoparietal cortex (Fig.9). Although these neurons possessed a pyramidally shaped soma and apical dendrites, the basal dendrites were difficult to identify. The striate cortex was negative.

**Day 10**

At this stage, the trigeminal and septal nuclei, thalamus, hypothalamus, ICP, and cerebellar layers were all characterized by a substantial decrease in positively stained cells. Within the caudate/putamen, a single small focus of positively stained round cells was visible. The frontoparietal cortex was negative for HSV1, but many large, positive pyramidal shaped neurons were present in the primary olfactory cortex (Fig.10). Additionally, the oculomotor nucleus showed an increase in the number of positively stained elements. The elements were round in shape, but morphologically, difficult to identify.
Fig. 9. Section through the frontoparietal cortex of an animal 8 days after inoculation. Pyramidal/fusiform shaped neurons within the external pyramidal layer are positively stained for HSV1; however no basal dendrites are visible. This may be due to incomplete impregnation of viral antigen. Section is lateral to the posterior cingulate gyrus. (B=-3.8 mm) Bar=50 μm. (See C-C’ in Fig.25).
Fig. 10. Section through the primary olfactory cortex of an animal 10 days after inoculation. HSV1 immunoreactive pyramidally shaped neurons lateral to the anterior cortical amygdaloid nucleus. As in Fig. 9, basal dendrites are difficult to identify. (B=-1.8 mm) Bar=50 μm.
Day 12

By this stage, very few areas remained visibly infected. The pyriform cortex was negative for HSV1, but a small focus of positively stained, small, dark, round cells was present in the cingulum. A few positively stained cells remained within the granular layer of the cerebellum, most of which were small and round in shape. What appeared to be a process and an elongated soma were also positively stained within the section (Fig.11).

Day 30

One month after inoculation, all brain areas were essentially negative for HSV1, with an occasional spotting in a few brain regions.

MHC Class I Antigen Expression

In control animals, MHC class I expression (OX18) was largely confined to vascular endothelial cells and a few small glial cells throughout the brain (Fig.12). The immunohistochemical signal for the glial cells was stronger in white matter than in grey with morphology consistent with that of resting microglia. These cells were multipolar with many fine spines and a slightly elongated soma.

Tissue examined at 3 days post-infection revealed no change in class I staining from that seen in control animals. By 6 days, however, a large number of cells characteristic of reactive microglia were positively stained for MHC class I. These cells were multipolar and star-shaped, with an elongated,
Fig. 11. Section through the cerebellar cortex (granular cell layer 1) of an animal 12 days after inoculation. HSV1 immunoreactive process (large arrow) and small, round, cells (small arrow) are evident. The round cells are approximately 5-10 μm in diameter with no visible processes. They are most likely leukocytes. (B=-10.3 mm) Bar=100 μm.
Fig. 12. Section through the VPM nucleus of the thalamus of a control animal 6 days after corneal scarification. MHC class I reactive blood vessels are evident throughout the tissue. (B=−3.8 mm) Bar=100 μm.
slightly swollen soma. The soma was approximately 15-20 μm in
length, with many swollen processes. Internal cellular structures
were difficult to identify due to the immunostaining.

Positively stained microglia were present in many brain
regions similarly positive for HSV1. At 8 days, the number of
positive microglia increased substantially and were largely
confined to HSV1 positive brain areas, with a few exceptions. Ten
and 12 days post-infection were characterized by class I positive
microglia throughout the entire brain. At 30 days, however, the
number of positive cells had decreased substantially, remaining
only in regions which had initially been heavily stained for
HSV1. Similar to staining patterns visualized for HSV1, class I
staining was largely ipsilateral at 6 days, progressing to a
bilateral distribution during the 8-12 day period, and returning
to an ipsilateral distribution at 30 days.

Day 6

Class I positive reactive microglia and endothelial cells
were present in the area of the spinal trigeminal nucleus
(Fig.13). Positive microglia were also present in molecular
layers 3-5 of the cerebellum as well as in the ICP. Darkly
stained, small, round cells were also evident in the ICP
(Fig.14).

Positively stained microglia were present within the lateral
hypothalamic area as well as in the median eminence.
Fig. 13. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. MHC class I positively stained microglia cells (large arrowhead) with typical microglia morphology (see text). A few MHC I immunoreactive blood vessels are also evident (small arrowhead). (B=-10.3 mm) Bar=50 μm.
Fig. 14. Section through the inferior cerebellar peduncle of an animal 6 days after inoculation. MHC class I immunoreactive small, round cells, with no visible processes are present. They are approximately 5-10 μm in diameter and are most likely leukocytes. (B = -10.3 mm) Bar = 50 μm.
Posterior and dorsal hypothalamic areas were negative. The medial geniculate nucleus (MGN) and superior colliculus both contained positive microglia, but no reactivity was noted in either the LGN or VPL/VMP nuclei. A few reactive microglia were present in the subiculum.

Positively stained microglia and endothelial cells were noted in the Edinger-Westphal nucleus, and, although the caudate/putamen was negative for class I, a few lightly stained microglia were present in the internal capsule (Fig.15). Cortical and lateral septal areas were largely negative for class I.

Day 8

A substantial increase in the number of class I positive microglia was noted in the aforementioned brain regions 8 days after inoculation. Additional brain areas containing positively stained microglia included posterior and dorsal hypothalamic areas, VPM, LGN, lateral habenular nuclei, and caudate/putamen. A large number of positively stained, small, round cells were also present in the posterior hypothalamic area. Class I positive microglia were also noted in the external pyramidal layer of the frontoparietal cortex (3,1,2) as well as in the cingulum. Focal areas of reactivity were also present in the lateral septal area (Fig.16).
Fig. 15. Section through the internal capsule of an animal 6 days after inoculation. MHC class I immunoreactive microglia are evident. (B=-3.8 mm) Bar=50 μm.
Fig. 16. Section through the lateral septal nuclei of an animal 8 days after inoculation. Note MHC class I immunoreactive microglia cell with elongated soma and multiple fine processes. (B=1.7 mm) Bar=50 μm.
Days 10 and 12

At 10 days, the entire brain was densely populated with class I positive microglia. A few focal patches of heavily stained microglia were present in the MGN of the thalamus (Fig.17). By 12 days, a slight decrease in the number of positively stained microglia was evident throughout the brain, but overall, the brain remained densely populated.

Day 30

A dramatic decrease in the number of positive microglia was clearly evident at this stage. Within the trigeminal nucleus and cerebellar layers, the level of reactivity was similar to that seen initially at 6 days. Some microglia remained in the ICP, but small, round cells were no longer visible. Decreased numbers of reactive microglia were also evident in the lateral hypothalamus, Edinger-Westphal nucleus, superior colliculus, and internal capsule. A few focal areas remained in the VPL/VPM nuclei and a few positive cells were present in the caudate/putamen, cingulum and lateral septal nuclei.

MHC Class II Antigen Expression

Control tissue exhibited far less MHC class II (OX6) than class I staining. Only a few class II (Ia) positive cells with typical resting microglia morphology were found. These cells were largely confined to white matter.
Fig. 17. Section through the thalamus of an animal 10 days after inoculation. A large focal area of MHC class I positive microglia are present within the MGN, lateral to the deep mesencephalic nuclei. Note relative density compared to background reactivity. (B=-6.3 mm) Bar=100 μm.
Three days following inoculation, brain tissue was negative for class II antigens. At 6 days, class II positive microglia were present in brain regions similarly positive for class I. Ia expression followed a pattern comparable to that seen in class I expression during the 8 to 12 day period. A dramatic decrease in Ia expression was evident at 30 days. Although class I and class II expression exhibited largely comparable distributions, some differences were evident. And similar to HSV1 and class I staining, class II expression was initially ipsilateral, followed in later stages by a bilateral distribution, and returning to an ipsilateral pattern by 30 days.

Day 6

Positively stained microglia and small, dark, round cells were present in the trigeminal nucleus (Fig.18). These microglia were slightly different morphologically from class I positive microglia, in that the class II positive microglia had fewer processes and these processes were less swollen. The differences noted between the class I and II positive microglia are likely due to differences in the quality of staining. The small, dark, round cells were approximately 5-10 μm in diameter with no visible processes. These cells were most likely leukocytes.
Fig. 18. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. MHC class II immunoreactive microglia. Note slight differences in morphology from MHC I positive microglia. These microglia possess fewer processes which are also less swollen relative to class I microglia. The disparity is likely due to differences in quality of staining. (B=-11.3 mm) Bar=50 μm.
**Day 8**

Both microglia and round cells increased in number within the trigeminal nucleus. The number of round cells within the ICP had also increased and small foci of Ia positive microglia were evident in both the granular and molecular layers (2-5) of the cerebellar cortex. Small foci of class II positive microglia were also noted in the posterior hypothalamic area, VPL/VPM nuclei, MGN, superior colliculus, and lateral septal nuclei. A few Ia positive microglia and small, dark, round cells were present in the internal capsule and a few positively stained microglia were seen in the caudate/putamen. Ia positive round cells were also noted in the Edinger-Westphal nucleus. A few reactive microglia were present in the frontoparietal cortex (3,1,2) and the cingulum.

**Days 10 and 12**

Similar to the class I distribution at 10 days, class II positive microglia were evident throughout the entire brain. A few focal areas of intense staining were noted in scattered midbrain nuclei (Fig.19). Very little change in the number of Ia positive microglia was evident at 12 days. A few areas, notably the pyriform cortex, olfactory tubercle, and hippocampus, contained slightly reduced numbers of reactive microglia.
Fig. 19. Section through the deep mesencephalic nuclei of an animal 10 days after inoculation. Note heavily stained focus of MHC class II immunoreactive microglia relative to background staining. Focus is medial to the MGN of the thalamus and lateral to the third nucleus. (B=-6.3 mm) Bar=50 μm.
Day 30

A substantial decrease in the number of positively stained microglia was evident throughout the brain. A few reactive microglia remained in the trigeminal nucleus, ICP, granular and molecular cerebellar layers 2-3, and in the rostral portion of the caudate. A single focus of Ia positive staining was present in the deep mesencephalic nuclei and a single positive microglial cell was noted in the MGN of the thalamus. Unlike the class I distribution at 30 days, a few Ia positive microglia remained in the optic chiasm and the forceps major of the corpus callosum.

Glial Fibrillary Acidic Protein (GFAP) Staining

Astrocytes were evident 3 days after inoculation. These cells were star-shaped with an oval shaped nucleus and well developed processes emanating from all sides (Fig.20). The time period from 6 to 10 days was characterized by astrocytic reactivity within HSV1 positive brain regions. At 12 days, tissue necrosis was present in the hypothalamus and cerebral peduncle. Necrotic areas were characterized by an absence of cells in the center, surrounded by a ring of darkly staining astrocytes. Although some necrosis was evident, there was no evidence of neuronal degeneration in either cresyl violet or hematoxylin and eosin stained sections. This may be due to variability in either staining or infection, or in both. No necrosis or cystic scarring was visible at 30 days.
Fig. 20. Section through the medial portion of the fifth nucleus of an animal 3 days after inoculation. Note star shaped GFAP positive resting astrocytes with well developed processes emanating from all sides. (B=-11.3 mm) Bar=50 µm.
Day 6

At this stage, changes characteristic of astrocytic reactivity were evident within those areas which were stained positively for HSV1. GFAP staining intensity was increased, nuclei were swollen, and the number of GFAP positive cells had increased over day 3. The somas were approximately 15-20 μm in length and astrocytic processes were slightly more tubular and wispy in appearance (Fig.21). Serial sections stained with cresyl violet revealed swollen astrocytic nuclei (Fig.22). With both GFAP and cresyl violet staining, internal cellular characteristics were difficult to identify. Astrocytes in HSV1 negative brain regions were not reactive.

Days 8 and 10

HSV1 staining was present in an increasing number of brain regions at this stage, and astocytic reactivity was noted in all of these areas. Staining intensity and the number of GFAP positive cells increased. A few focal areas of necrosis were present within the lateral hypothalamic area and the cerebral peduncle. The necrotic areas were devoid of all parenchymal elements and were surrounded by a darkly stained astrocytic margin (Fig.23).

Days 12 and 30

At 12 days, a decrease in the staining intensity and number of GFAP positive cells was evident in all brain regions except for the trigeminal nucleus, molecular cerebellar layers 3-5, and the ICP. These areas appeared as they did at 10 days. Small
Fig. 21. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. GFAP positive reactive astrocytes possess an enlarged and irregularly shaped soma with wispy processes (arrowheads). These characteristics are consistent with those described for reactive astrocytes (see text, Mori and Leblond, 1969). (B=-11.3 mm) Bar=50 μm.
Fig. 22. Section through the medial portion of the fifth nucleus of an animal 6 days after inoculation. Cresyl violet stain reveals astrocytes to be swollen and granulated (arrowheads). (B=-11.3 mm) Bar=50 μm.
Fig. 23. Section through the cerebral peduncle of an animal 12 days after inoculation. A large necrotic area (arrowheads) is evident with an absence of cells in the center, surrounded by a ring of darkly stained astrocytes. Necrotic area is dorsal to the pontine nuclei and ventral to the medial lemniscus. (B = -6.8 mm) Bar = 200 μm.
necrotic areas were visible in the cingulum, MGN, and caudate/putamen. A substantial decrease in staining intensity and cell number throughout the brain was evident at 30 days. A few reactive astrocytes remained in the VPL nucleus of the thalamus, but little necrosis or cystic scarring was visible in any area of the brain.

**Double Immunostaining**

Many of the cells which stained positively for class I and Ia antigens exhibited classical reactive microglia morphology. Some cells, however, were star-like in appearance, closely resembling astrocytes. In order to help clarify the cell populations expressing MHC antigens, tissue was doubly stained with GFAP for astrocytes and MHC I or II. In all sections examined (from all time periods), double staining revealed two separate cell populations. There was no overlap between astrocytes (GFAP+) and MHC class I or II positive cells (Fig.24).
Fig. 24. Section through the VPM thalamic nucleus of an animal 8 days after inoculation. GFAP (brown) and MHC class II (purple) double staining reveals astrocytes to be negative for MHC glycoproteins. (B=-4.3 mm) Bar=50 μm.
DISCUSSION

In the present study, MHC expression was examined in the rat CNS following a herpes viral infection. To summarize briefly predictions based on the literature, it seemed likely that HSV1 reactivity would be noted initially in the trigeminal nucleus, that the infection would spread to other brain regions, and that both neurons and glial cells would be infected.

Antibodies against MHC class I and II antigens were used to examine the brain regions, and cell types within those regions, which express MHC molecules. It seemed likely that MHC expression would be found within the CNS and that the cell type(s) expressing MHC antigens would be non-neuronal. Double immunostaining could clarify the antigen presenting cell types. To a large extent, the results of the present study closely followed the predictions.

HSV1

Behavioral changes characteristic of an encephalitis were evident 6-7 days after inoculation. The virus was initially found in the fibers and sensory nucleus of the fifth nerve and progressed rapidly to other brain regions. These early stages of infection clearly demonstrated cell to cell spread of the virus. Efferent projections from the trigeminal nucleus have been documented (Fig.25).
Trigeminal Pathways

Position of Trigeminal Tracts

Somatotopy in SpTT and SpTNu

Input from Cranial Nerves 7, 9, 10

(From Haines, 1987)
Fig. 25 Efferent Projections from the Trigeminal Nucleus

Abbreviations

AbdNu Abducens Nucleus
ALS Anterolateral System
CC Crus Cerebri
CSNu Chief Sensory Nucleus
DTTr Dorsal Trigeminothalamic Tract
FacNu Facial Nucleus
HyNu Hypoglossal Nucleus
IC Internal Capsule
Man.V Mandibular Division of Trigeminal Nerve
Max.V Maxillary Division of Trigeminal Nerve
OpTh.V Ophthalmic Division of Trigeminal Nerve
RB Restiform Body
RNu Red Nucleus
SN Substantia Nigra
SpTNu Spinal Trigeminal Nucleus
SpTT Spinal Trigeminal Tract
TriMoNu Trigeminal Motor Nucleus
VPL Ventral Posterolateral Nucleus of Thalamus
VPM Ventral Posteromedial Nucleus of Thalamus
VTTr Ventral Trigeminothalamic Tract

(From Haines, 1987)

Not Shown in Diagram:

Crossed projections to the

- Caudate/Putamen (Yasui, 1987)
- Cerebellum (Brodal, 1981)
- Cornea (Carpenter et al., 1983)
- Dorsal Motor Nucleus of X (Carpenter et al., 1983)
- Nucleus Ambiguus (Carpenter et al., 1983)
- Nucleus of Solitary Tract (Menetrey et al., 1987)
- Superior Salivatory Nucleus (Carpenter et al., 1983)
HSV1 immunoreactivity was clearly visible within all projection areas of the sensory nucleus of cranial nerve V by 8 days post-inoculation. Staining was bilateral at this stage in these brain regions except in the frontoparietal cortex (somatosensory), where staining was solely contralateral (Fig. 9). Efferent projections from positively labeled cells within the VPM nucleus of the thalamus to this cortical region, explain the presence of HSV1 immunoreactivity in this area (Fig. 7). Within the inferior cerebellar peduncle, staining was ipsilateral at 6 days but had become bilateral at 8 days, suggesting bilateral progression of the virus to the cerebellum from the trigeminal nucleus (spinal portion).

Afferent projections to the spinal trigeminal nucleus originate contralaterally from the medial and descending vestibular nuclei, perihypoglossal nuclei, and motor cortex (Brodal, 1981; Walberg et al., 1985). HSV1 immunoreactivity was clearly visible in the ipsilateral trigeminal axons as well as bilaterally in the superior, medial and descending vestibular nuclei, and perihypoglossal nuclei by 8 days post-inoculation. There was no visible staining evident within the frontoparietal motor cortex at any stage. Lack of staining in this region may be the result of variability in either staining or infection, or in both.

A number of brain regions were visibly infected which reportedly do not receive either projections from or send afferents to the spinal trigeminal nucleus. Extensive HSV1 immunoreactivity was noted in the hypothalamus and moderate
levels were noted in the septal area. A small number of positively labeled cells were evident within the anterior olfactory nucleus which likely reflects entry of the virus into the nasal pathway via the tear duct. Once inside the olfactory pathway, the virus most likely spread to nearby limbic structures, such as septal nuclei and primary olfactory cortex (Fig. 10).

These results suggest that, although herpes virus travels primarily via retrograde and to a lesser degree anterograde axonal transport, other modes of travel must be considered. The virus might bud into the extracellular space allowing entry into the CSF; however little evidence of HSV1 staining was present around ventricular areas. A blood-borne infection from the periphery must also be considered. Peripheral organs (spleen, thymus, liver) were negatively stained for HSV1 but this might indicate either an undetectable level of virus or lack of a blood-borne infection. The widespread distribution of HSV1 immunoreactivity during the later stages of infection suggests that the virus was traveling via a number of routes, including undiscovered afferent and efferent spinal trigeminal connections, as well as secondary and tertiary connections.

In a number of tissue sections, neurons as well as small, round cells were positively stained for HSV1. The size, shape, and time of appearance of the round cells were consistent with a leukocyte profile. Leukocytes are known to enter the tissue during early stages of the infection (Johnson, 1982). Additionally, lack of visible processes and the non-linear
distribution of these cells (oligodendrocytes often have a linear
distribution) suggested that they were not glial cells.
Consistent with these results, other studies have demonstrated
infected neurons following HSV1 inoculation (Lycke et al., 1984,
Vahlne et al., 1978, Ugolini et al., 1989). In contrast to the
present study, however, other investigators have reported the
presence of infected glial cells following inoculation (Lycke et
al., 1984, Vahlne et al., 1978). This discrepancy may be due to
the inability to identify clearly cell types with immunostaining
and the possibility of false negatives following staining.

Later stages (days 12 and 30) with little or no positive
HSV1 staining, likely reflected either the disappearance of
active virus or the presence of latent virus which the antibody
was unable to recognize. It should be noted that each animal in
these latter two time periods had recovered from the encephalitis
and appeared healthy prior to being sacrificed. It is
conceivable, therefore, that these animals may not have suffered
as severe an infection as the other animals. Additionally, there
was evidence of necrosis 12 days after inoculation but no
evidence of necrosis at 30 days. It is unlikely that necrosis
would have disappeared within this time period.

MHC Antigens

Until recently, lack of MHC expression in the CNS was
considered evidence in support of immunological privilege. As
reviewed in the introduction, however, in vitro and in vivo
studies provide evidence indicating that MHC expression does
occur under appropriate conditions. This study clearly demonstrated such expression in virally infected rat brain.

MHC positive reactive microglial cells were multipolar and star-shaped, with an elongated, slightly swollen soma. They were approximately 15-20 µm in diameter, with many swollen processes. This profile is consistent with that for reactive microglia as originally described by del Rio Hortega, (1919). Using the silver carbonate method, del Rio Hortega described the reactive microglia as uni- bi-, or multipolar with an elongated or triangular soma. This cell type progresses through a number of stages involving swelling of the soma and processes as well as vesiculation of the cytoplasm. Extensions are gradually retracted and numerous membrane-bound phagosomes become evident. The cell eventually becomes amoeboid or round in shape with a foamy cytoplasmic appearance. Although internal cellular structures were not visible with immunostaining, the cell type described in the present study is more than likely a microglial cell. Reproducibility of staining from rat to rat within groups sacrificed at the same time period was highly consistent.

MHC class I and II positively stained microglial cells were evident in a number of brain regions similarly positive for HSV1. Differences in staining between class I and II were apparent 6 days after inoculation. Thalamic and hypothalamic nuclei, as well as the superior colliculus, subiculum, and Edinger-Westphal nucleus contained class I but not class II positively labeled microglia at 6 days. The reasons why class II expression lagged behind class I at this stage, are not clearly understood. By day
8, however, both MHC antigens were equally distributed throughout infected brain regions. Small, round, MHC positive cells were noted, particularly in the area of the trigeminal nucleus, during days 6 and 8. Serial sections stained with cresyl violet revealed a large number of these cells to be polymorphonuclear (PMN) cells with characteristic multi-lobed nuclei.

Interestingly, although a large number of brain areas were HSV1 positive at 10 days, the entire brain did not show such staining but it did show class I and II expression. Additionally, after positive HSV1 staining was no longer visible (day 12), class I and II positive microglia continued to populate the brain. There were, however, a few brain areas at this stage which had a slight decrease in the number of positively staining microglia. Both MHC antigens were still visible in a few select brain regions 30 days after inoculation. Notably, class II positive microglia were visible within the optic chiasm, which was not positive for HSV1 at any stage. One possible explanation for this discrepancy may be the limitations of the technique. Immunostaining can produce false negatives. And, as mentioned earlier, this animal may have suffered minimal infection, which could also lead to discrepancies in staining.

These results suggest that between 8 and 10 days, MHC expression was no longer confined to visibly infected areas, indicating a systemic CNS immune response was underway. This vigorous response was still present at 12 days, although most of the virus was apparently no longer active in this presumably recovered rat. Signs of immune reactivity were still visible 30
days following inoculation. The time pattern for MHC expression in the present study was similar to other CNS insults (Akiyama et al., 1988). Although previous studies have not examined MHC expression in the CNS following HSV1 infection, a few studies have reported MHC positive reactive microglia following various insults (Akiyama et al., 1988, Antoniou et al., 1987, Hickey et al., 1987).

Functionally, peripheral MHC class I expression is known to be involved in viral immunity (Lampson, 1987). Cell-mediated cytolysis of virally infected cells is thought to occur through three distinct mechanisms: cytotoxic T cells, natural cytotoxicity, and antibody dependent cell-mediated cytotoxicity (Stites et al., 1987). During a viral infection, viral antigens are "presented" on the cell surface of the infected cell in combination with a MHC class I antigen (Stites et al., 1987). This combination is then recognized by cytotoxic T-lymphocytes possessing receptors specific to this combination. Recognition then initiates cell-mediated lysis (CML) of the infected cell. This allows for the release of viral progeny into the extracellular space which infect glial cells and initiate further immunological responses. T-helper cells and antibody producing plasma cells play an important role in eliminating the virus during this stage. Class I expression is clearly instrumental in the immunological defense process during peripheral HSV1 infection. Class I expression in the CNS may be indicative of similar immune reactivity.
The functional significance of peripheral class II expression during HSV1 infection differs from that of class I expression (Lampson, 1987). Class II antigen presentation is also integral to the recognition process, but recognition is followed by a series of events functionally different from class I antigen presentation. Antigen presentation occurs when a foreign antigen is consumed by an antigen presenting cell (APC), often a macrophage, resulting in presentation of pieces of the foreign particle on the cell surface in combination with a class II molecule. This combination is then recognized by T-helper lymphocytes possessing receptors specific for this combination. Once recognition occurs, an immune cascade begins. T-helper cells initiate cloning of additional T-helper and T-cytotoxic cells, as well as stimulating B-cells to differentiate into antibody-producing plasma cells. Large amounts of antibody specific to the foreign antigen are produced in an attempt to eliminate the invading antigen.

Humoral antibody production is demonstrable in both peripheral and central HSV1 infection, but offers very little protection (Johnson, 1982, Lampson, 1987). Due to its ability to travel transsynaptically, HSV1 is largely protected from antibody in the surrounding extracellular fluid. However, it is apparent that MHC class II expression is an active component during viral infection. Based on the known functions of both class I and II MHC antigens in the peripheral immune response, expression of these molecules in the CNS suggests a similar pattern of immune reactivity.
GFAP

GFAP staining revealed pathological changes in astrocytes characteristic of a viral infection (Johnson, 1982, Viken et al., 1978). Staining intensity was increased, nuclei were swollen, and the number of GFAP positive cells had increased. The somas were approximately 15-20 μm in diameter and astrocytic processes were slightly wispy in appearance. Reactive astrocytes, as described by Mori and Leblond, show an enlargement of the soma with a slightly enlarged, eccentrically located nucleus (Mori and Leblond, 1969). The processes lose their sheet-like expansions and become tubular and wispy in appearance. Glial fibrillary acidic protein is also greatly increased. Although internal cellular changes were not clearly visible in the cells identified in the present study, their morphology is consistent with that of reactive astrocytes.

These changes in astrocyte morphology were evident in areas of HSV1 reactivity and persisted through day 12. At 10 days, small necrotic-like areas could be seen in a few brain regions and some necrosis was evident at 12 days. No necrosis or cystic scarring was visible at 30 days, but MHC reactive microglia were present, suggesting some level of immune activity. The lack of necrosis and cystic scarring at this stage, however, suggests that the infection in this animal may have been minimal.

Microglia

In the present study, the main CNS cell type staining positively for both MHC antigens was the reactive microglia-like cell. This was morphologically determined based on classical
descriptions of this cell type by del Rio Hortega and Penfield (del Rio Hortega, 1919, Penfield, 1925). The two main issues surrounding this controversial cell type are antigen presentation and the origin of these cells.

As mentioned earlier, antigen presentation is essential for initiation of the immune cascade, or CMI. Peripherally, APCs consist largely of macrophages/monocytes and a few T and B lymphocytes. The question arises as to which cell or cells in the CNS are performing this function. Studies involving in vitro work with cultured astrocytes (Barna et al., 1987, Fontana et al., 1984, Male et al., 1987, Massa et al., 1986, 1987) and analysis of post-mortem MS brain tissue (Hofman et al., 1986, Traugott, 1985, 1987) have reported class II positively stained astrocytes. The authors state that these results suggest that astrocytes are serving the function of antigen presentation in the CNS. Double staining techniques were used in the present study in an attempt to clarify this issue. Antibodies against both GFAP (astrocytes) and MHC class I or II determinents (OX18, OX6) were used in a double staining procedure to examine the question of antigen presentation. Multiple sections revealed no overlap in positive reactivity for GFAP and OX18 or OX6. Two distinct cell populations were present, indicating astrocytes were not expressing class I or II antigens on their surface and therefore did not possess antigen presentation capabilities. These results have been replicated in our laboratory with both MS and Alzheimer's brain tissue, and after KA lesioning in the rat CNS (Akiyama et al., 1988, McGeer et al., 1987a, 1987b). The cells
which did stain positively for MHC class II were morphologically identified as reactive microglia. Many studies have demonstrated that MHC class I and II expression is a function of immune cells and not a function of ectodermally derived glial cells.

Although currently a controversial cell type, microglia possess phagocytic capabilities and are thought to be the brain macrophage (Akiyama et al., 1988, Jordan et al., 1988). Peripheral macrophage antigen presentation together with evidence of class II expression on microglia in the CNS suggests functional similarities between these two cell types. However, the origin of these brain macrophages remains elusive.

**Origin of Microglia**

Microglia are thought to be either part of the glial family (neuroectodermal origin; Fujita, 1981, Matsumoto et al., 1987, Schelper et al., 1986) or part of the mononuclear phagocyte system (hematogenous origin; Akiyama et al., 1988, Dolman, 1985, Esiri et al., 1986, Giulian, 1987, Hickey et al., 1988, Imamoto et al., 1978, Kitamura et al., 1972, 1980, Murabe et al., 1981, Oehmichen, 1980, Wood et al., 1979). Although during disease processes involving compromise to the blood-brain barrier, monocytes may enter the tissue and differentiate into macrophages, it is not clear whether an endogenous glial macrophage performing the same function also exists. A number of studies attempting to answer this question have met with inconclusive results. One study, however, provides some important evidence in support of a hematogenous origin (Akiyama et al., 1988). In this study, CNS tissue from KA lesioned rats was
stained with antibodies against both MHC class II (OX6) and leukocyte common antigen (LCA). The results showed that microglia and infiltrating macrophages were positively stained for both OX6 and LCA. Glial cells of neuroectodermal origin (astrocytes, oligodendrocytes) did not stain for either of the two antibodies in all sections examined. The authors suggest that the results indicate differing gene expression between microglia and neuroglia and similarities in gene expression between microglia and macrophages. Although these results lend support for the hematogenous origin of microglia, the controversy still remains.

Summary

The present study demonstrated MHC expression in the rat CNS following a herpes virus infection. A detailed map of infected brain areas as well as a comprehensive time course of MHC expression were outlined. An initial PMN infiltrate was followed by elevated MHC antigen expression on microglia cells in and around HSV1 positive brain areas. During later stages of infection, the entire brain was densely populated with reactive microglia, even in areas negative for HSV1. Astrocytes showed typical pathological changes, but did not express MHC antigens at any time. A few small foci of necrosis were evident at 10 and 12 days, but no evidence of necrosis was visible at 30 days post-inoculation, suggesting minimal infection in this rat. This sequence of events conceivably reflects CMI and a systemic CNS immune response, resulting in phagocytosis of the virus with latent virus remaining in the brain. The immune system was
apparently unable to overcome the viral infection in animals which did not recover from the encephalitis.

The results from the present study are important for two main reasons. Most importantly, MHC expression on endogenous CNS elements following viral infection suggests the capacity for antigen presentation and the initiation of CMI. These results indicate that MHC molecules play an essential role in CNS viral clearance in a manner similar to the periphery. Secondly, it is important to understand further how the CNS responds to viral infection. A clear understanding of the CNS response to a viral infection may provide information necessary for therapeutic intervention, as well as providing additional information regarding the etiology of certain CNS diseases.

The Future

The results gleaned from the present study suggest further examination and manipulation of the relevant issues. Based on the limitations of immunohistochemistry, such as false negatives and difficulty in distinguishing intracellular elements or stained structures, electron microscopy (EM) would provide definitive evidence of the findings put forth in the present study. Although also somewhat limited, EM would aid in clarification of cell types expressing MHC antigens as well as in identification of viral particles throughout the brain.

To complete the picture, examination of other important immunological markers may prove informative. Receptors for lymphokines, such as interleukin-2, and for the various lymphocytes, such as T cell subsets and B cells, can be
immunohistochemically labeled and examined for their role in the immune response to viral infection. In this same vein, human post-mortem tissue from Parkinson's and Alzheimer's patients has been examined for these immunological markers in this laboratory (Itagaki et al., 1987, 1988, McGeer et al., 1988). The results suggest some form of immunologic disturbance prior to death, yet evidence for viral infection is still being sought. Post-mortem examination of herpes encephalitis brain tissue may provide important information regarding the immunological processes involved in attempting to clear herpes virus from the CNS.

Finally, manipulations during the acute infection may provide useful information for therapeutic intervention. Such manipulations might involve the use of interleukins or interferons to accelerate or attenuate the immune response. Additionally, pharmacological manipulation of the blood-brain barrier may also have an effect on the course of the infection (Mokhtarian et al., 1984). It is important to examine both basic scientific questions as well as clinically related questions in order to understand fully the processes involved in viral infections of the CNS and their possible relation to neurological disorders.
Bibliography


**Honors/Awards/Publications**

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**Publications**


