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Herpes simplex virus type 1 encephalitis is associated with elevated levels of F_2-isoprostanes and F_4-neuroprostanes

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To better understand the pathogenesis of herpes simplex virus type 1 (HSV-1) infections of the nervous system, concentrations of F_4-neuroprostanes (F_4-NP) and F_2-isoprostanes (F_2-IP) in the murine brain were determined following intracerebral inoculation of HSV-1 or normal saline. F_4-NP are highly selective, quantitative markers of neuronal oxidative damage, while F_2-IP are markers of oxidative damage to brain tissue not limited to a certain cell type. In contrast to saline-treated control animals, HSV-1-infected animals developed encephalitic symptoms associated with severe inflammation, widespread HSV-1 protein expression, and significantly elevated F_4-NP and F_2-IP levels in the brain. Survivors of acute HSV-1 infection showed no encephalitic symptoms 2 to 3 weeks following virus inoculation. Brain tissue derived from mice euthanized 2 month after virus inoculation demonstrated expression of HSV-1 latency-associated transcripts without detectable HSV-1 protein expression. However, brain tissue from these animals showed focal chronic inflammation, moderately elevated F_2-IP levels, and normal levels of F_4-NP. These observations provide novel biochemical evidence that oxidant tissue injury is a mechanism underlying neuronal damage during acute HSV-1 encephalitis and suggest that oxidative damage to tissue may continue in the mammalian brain until at least several weeks after recovery from the symptomatic phase of HSV-1 infection. *Journal of NeuroVirology* (2002) **8,** 295–305.

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Introduction

Herpes simplex virus type 1 (HSV-1) is an important pathogen infecting the vast majority of humans worldwide (reviewed in Whitley, 2001). Following primary infection of mucocutaneous sites, HSV-1 travels by axonal routes to the nervous system where it can cause encephalitis, or much more commonly establishes a latent infection in neurons (reviewed in Skoldenberg, 1996; Steiner, 1996; Whitley, 2001). HSV-1 latent infection of the peripheral nervous system (PNS) affects a majority of humans by adulthood (Liedke *et al*, 1993; Baringer and Pisani, 1994; Schultz *et al*, 1998). Reactivation of HSV-1 from neurons can lead to recurrent mucocutaneous disease and occasionally encephalitis (reviewed in Skoldenberg, 1996; Whitley, 2001). As a consequence of primary infection, HSV-1 genomic DNA is also present in the central nervous system (CNS) of a significant

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proportion of the adult human population (Fraser *et al*, 1981; Liedke *et al*, 1993; Baringer and Pisani, 1994; Itzhaki *et al*, 1997). However, based primarily on observations made using animal models of HSV-1 pathogenesis, it is thought that viral reactivation does not occur in the CNS (Cabrera *et al*, 1980; Steiner *et al*, 1994). Although encephalitis develops in only a small minority of primary and reactivation HSV-1 infections, HSV-1 is the most common cause of sporadic encephalitis in the United States. The molecular mechanisms by which HSV-1 damages the nervous system are not well understood.

Injury mediated by free radicals, most notably by reactive oxygen species (ROS) and reactive nitrogen species (RNS), is an important common pathway of tissue damage for a wide variety of acute and chronic pathologic processes (reviewed in Halliwell and Gutteridge, 1999). ROS and RNS induced by viruses and reactive inflammatory responses can serve as messenger molecules that modulate cellular activation state and influence viral growth. Tissue damage mediated by ROS and RNS is increasingly recognized as an important mechanism by which viral infections can damage the host (reviewed in Schwartz, 1996; Peterhans, 1997a, 1997b; Akaike *et al*, 1998). Free radical-mediated injury also is a mechanism by which viral infections can damage the nervous system. Neural damage induced by free radicals has been reported as a consequence of human immunodeficiency virus infection of humans (Boven *et al*, 1999), reovirus infection of mice (Valyi-Nagy *et al*, 1999), and murine leukemia virus infection of rats (Wilt *et al*, 2000).

ROS and RNS also play important roles in HSV-1 pathogenesis. HSV-1 infection has been found to cause free radical-mediated injury in cultured cells (Palu *et al*, 1994) and in the murine lung (Adler *et al*, 1997). HSV-1 infection induces nitric oxide (NO) production in differentiated monocytes and macrophages in culture (Lopez-Guerrero and Alonso, 1997; Fujioka *et al*, 2000) and in the nervous system of rats (Fujii *et al*, 1999). Infection of ocular, lung, and nervous system tissues of mice is associated with the expression of the inducible form of NO synthetase (iNOS) and the release of cytokines including tumor necrosis factor (TNF)-α from inflammatory cells (Koprowski *et al*, 1993; Adler *et al*, 1997; Shimeld *et al*, 1997; Daheshia *et al*, 1998; Meyding-Lamade *et al*, 1998; Fujii *et al*, 1999; Nucci *et al*, 2000). NO and TNF-α have anti-HSV-1 activity and can either generate potent oxidizing byproducts directly (NO) or induce free radical-mediated injury indirectly through membrane signaling (TNF-α) (Matthews *et al*, 1987; Feduchi *et al*, 1989; Rossol-Voth *et al*, 1991; Croen, 1993; Karupiah *et al*, 1993; Sanchez-Alcazar *et al*, 2000). HSV-1 infections are associated with increased activity of antioxidant enzymes and depletion of the antioxidant glutathione, providing further evidence that HSV-1 infection is associated with oxidative stress (MacLean *et al*, 1998; Nucci *et al*, 2000). In concordance with this idea, therapy with the antioxidant glutathione reduces the severity of HSV-1 induced eye disease in mice (Nucci *et al*, 2000).

We have studied experimental HSV-1 infections of mice to define the role of free radical-mediated tissue injury in HSV-1 neuropathogenesis. As in humans, infection of peripheral tissues in mice leads to viral replication in epithelia, spread of virus by neural pathways from the PNS to the CNS, and occasional encephalitis depending on viral strain and dose (reviewed in Wagner and Bloom, 1997; Roizman and Knipe, 2001). Direct intracerebral (i.c.) inoculation of HSV-1 also leads to encephalitis. Animals surviving acute HSV-1 infection do not demonstrate signs or symptoms of encephalitis, and infectious virus is not detectable in the nervous system. However, HSV-1 establishes latency in these animals in neurons of the PNS and HSV-1 DNA remains detectable in the CNS (Cabrera *et al*, 1980; Steiner *et al*, 1994). Latent HSV-1 infections of neurons in mice show features similar to those seen in humans: viral genomic DNA is present in neuronal nuclei, abundant viral gene expression is limited to the latency-associated transcripts (LATs), and viral proteins or infectious virus are not detectable (reviewed in Wagner and Bloom, 1997; Roizman and Knipe, 2001). In addition, HSV-1 latency in the nervous system of mice is associated with persistent, chronic inflammation, persistently elevated levels of cytokines, and iNOS activity (Koprowski *et al*, 1993; Cantin *et al*, 1995; Meyding-Lamade *et al*, 1998; Chen *et al*, 2000; Valyi-Nagy *et al*, 2000).

Previous studies in our laboratory using a murine HSV-1 neuropathogenesis model and immunohistochemical detection methods of oxidant tissue injury have provided evidence that free radical-mediated tissue damage is a mechanism by which both acute and latent HSV-1 infections can cause damage in the nervous system (Valyi-Nagy *et al*, 2000). In the present study we used quantitative biochemical detection methods of oxidant tissue injury to test the hypothesis that oxidant brain injury occurs during HSV-1 infection of the murine nervous system. We have shown previously that free radical damage to the brain can be sensitively and accurately quantified by measuring chemically stable oxidative damage products of arachidonic acid (AA) and docosahexaenoic acid (DHA), F_2-isoprostanes (F_2-IP) and F_4-neuroprostanes (F_4-NP), respectively (Morrow and Roberts, 1997; Roberts *et al*, 1998). AA is relatively evenly distributed in the brain with similar concentrations in gray and white matter and within glia and neurons. Thus, measurement of F_2-IP is a reflection of oxidative damage to the entire brain, a limitation shared by all other quantitative methods of determining oxidative damage to the brain, except F_4-NP. Unlike AA, DHA is highly concentrated in neuronal membranes to the exclusion of other cell types. Moreover, we have shown that F_4-NP are by far the most abundant products of this pathway in

the brain (Roberts *et al*, 1998). Thus, measurement of F_4-NP provides a highly selective, quantitative window into neuronal oxidative damage.

We found that levels of F_2-IP and F_4-NP are markedly elevated during acute HSV-1 encephalitis and that levels of F_2-IP remain moderately elevated until several weeks after recovery from the acute, symptomatic phase of infection. These observations provide novel biochemical evidence that oxidant tissue injury is a mechanism underlying neuronal damage during acute HSV-1 encephalitis and suggest that oxidative damage to tissue may continue in the mammalian brain following recovery from the symptomatic phase of HSV-1 infection.

Results

HSV-1 infection of mice

To better understand the pathogenesis of oxidant neural injury during HSV-1 encephalitis, 4- to 6-week-old female BALB/c mice were inoculated i.c. in the left cerebral hemisphere with 1×10^3 or 1×10^5 plaque-forming units (PFU) of HSV-1 strain 17^+ or normal saline (mock-infection). The i.c. route of inoculation was chosen to provide synchronous, widespread CNS infection. Saline-inoculated animals demonstrated no disease symptoms following recovery from anesthesia. All HSV-1-infected animals developed encephalitic symptoms during the first two weeks following HSV-1-inoculation, irrespective of viral dose. These symptoms included agitation, hunched posture, ruffled fur, lethargy, circling behavior, and paralysis. None of the mice inoculated with 1×10^5 PFU survived beyond 2 weeks following infection; however, occasional mice survived HSV-1 infection following inoculation with 1×10^3 PFU. These surviving animals demonstrated no encephalitic symptoms beyond 2–3 weeks postinoculation and showed normal development.

Acute HSV-1 infection is associated with severe inflammation, widespread HSV-1 protein expression, and significantly elevated F_2-IP and F_4-NP levels in the murine brain

Hematoxylin and eosin-stained sections of tissues derived from the right brain half of mice euthanized 5 to 8 days after i.c. HSV-1 inoculation invariably demonstrated evidence of meningoencephalitis. Histologic sections showed inflammatory cell infiltrates in the brain parenchyma and meninges. Inflammatory changes primarily involved the brain stem, the diencephalon, the cerebral cortex, and the olfactory bulb. Perivascular and parenchymal inflammatory infiltrates consisted mostly of mononuclear inflammatory cells, but occasional necrotizing foci associated with infiltrates rich in neutrophils also were observed (Figure 1A, Table 1). Inflamed foci demonstrated widespread expression of HSV-1 proteins (Figure 1E, Table 1) and LAT (Figure 2A, Table 1). HSV-1 immunoreactivity and LAT expression primarily involved cells with the morphologic features of neurons. Sections derived from the right brain half of saline-inoculated animals euthanized 5 days following inoculation demonstrated normal histology and no evidence of HSV-1 protein or LAT expression (Figures 1B, 1F, 2B, Table 1).

Apoptotic cell death in the brain of HSV-1-infected and mock-infected mice was determined by detection of apoptosis-associated DNA strand breaks in tissue sections using a terminal deoxynucleotidyl transferase (TdT)-mediated DUTP-digoxigenin nick end-labeling (TUNEL) assay. TUNEL analysis of brain tissues derived from mice euthanized 5 to 8 days after HSV-1 inoculation demonstrated numerous TUNEL-positive cells that were present primarily in inflamed brain areas (Figure 3A). Morphologically, TUNEL-positive cells consisted of both neurons and nonneuronal cells. CNS tissues derived from uninfected mice and from mice euthanized five days following mock-infection with saline contained no TUNEL-positive cells (Figure 3B). These findings suggest that acute HSV-1 encephalitis is associated with apoptosis, in agreement with previous reports (Geiger *et al*, 1995, 1997; Pelosi *et al*, 1998; Valyi-Nagy *et al*, 2000).

To determine whether oxidant injury occurs during acute HSV-1 infection of the murine CNS, concentrations of F_2-IP and F_4-NP in the brain of mice were determined following i.c. inoculation of HSV-1 or normal saline (Figures 4, 5, Table 1). F_2-IP levels did not increase in the left, inoculated brain half of saline-treated animals euthanized 5 days after inoculation relative to F_2-IP levels detected in the brain of untreated mice (Figure 4, Table 1). This finding indicates that the minor trauma associated with inoculation does not alter F_2-IP levels. Brain tissues of mice euthanized 5 to 8 days after HSV-1 inoculation demonstrated significantly elevated F_2-IP levels relative to saline-inoculated ($P < 0.01$) and untreated controls ($P < 0.01$) (Figure 4, Table 1). Brain tissues of mice euthanized on days 5 to 8 after inoculation with HSV-1 also showed significantly elevated F_4-NP levels relative to saline-inoculated controls ($P < 0.02$) (Figure 5, Table 1). These findings demonstrate that i.c. inoculation of HSV-1 into mice leads to generalized encephalitis associated with oxidant brain injury that involves neurons.

Evidence of focal inflammation and moderately elevated F_2-IP levels in the murine brain several weeks after recovery from symptomatic HSV-1 encephalitis

To determine whether inflammation, evidence of HSV-1 infection, or oxidant tissue injury are detectable in the CNS of mice that survive the acute, symptomatic phase of HSV-1 infection, groups of mice were euthanized 60 days after i.c. inoculation with either HSV-1 or normal saline. Hematoxylin and eosin-stained sections of tissues derived from the right brain half of five of six mice euthanized 60 days after i.c. HSV-1 inoculation demonstrated

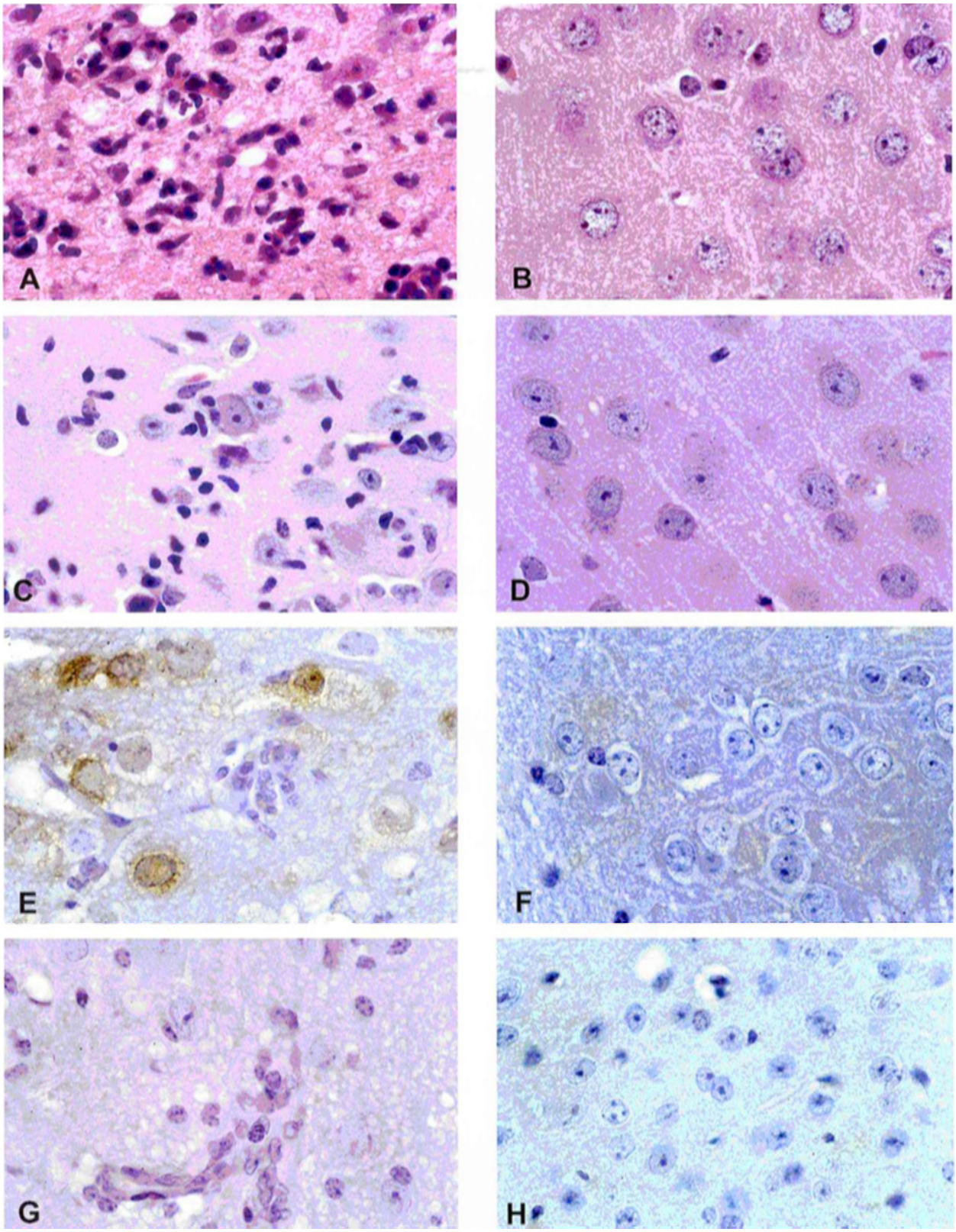

Figure 1 Inflammation and HSV-1 protein expression in the right cerebral hemisphere of BALB/c mice following i.c. inoculation of either HSV-1 or normal saline (mock-infection) into the left cerebral hemisphere. Hematoxylin and eosin-stained sections (**A**) 5 days following inoculation of 10^5 PFU of HSV-1. (**B**) 5 days following mock-infection. (**C**) 60 days following inoculation of 10^3 PFU of HSV-1. (**D**) 60 days following mock-infection. Detection of HSV-1 protein expression using a polyclonal anti-HSV-1 antiserum. (**E**) 5 days following inoculation of 10^5 PFU of HSV-1. (**F**) 5 days following mock-infection. (**G**) 60 days following inoculation of 10^3 PFU of HSV-1. (**H**) 60 days following mock-infection. (magnification 400×).

Table 1 HSV-1 protein and LAT expression and inflammation in the right brain half and F_2-isoprostane and F_4-neuroprostane levels in the left brain half of adult BALB/c mice 5 to 8 days and 60 days following intracerebral (i.c.) inoculation of HSV-1 strain 17^+ or normal saline

		No. of animals pos/No. of animals tested for:			*Brain concentration of:*	
Inoculum (i.c.)	*Time of euthanasia (days after i.c. inoculation)*	*HSV-1 protein expression*[1]	*HSV-1 LAT expression*[2]	*Inflammation*	*F_2-isoprostanes (ng/g) ± SD*	*F_4-neuroprostanes (ng/g) ± SD*
None	NA[3]	0/6	0/6	0/6	2.77 ± 0.28 $n = 9$	ND[4]
Saline	5	0/3	0/3	0/3	2.70 ± 0.15 $n = 6$	60.2 ± 8.1 $n = 8$
HSV-1 (10^3–10^5 PFU)	5–8	10/10	5/5	10/10	$5.82 \pm 1.02^*$ $n = 8$	$132.8 \pm 16.0^*$ $n = 5$
Saline	60	0/5	0/5	0/5	2.52 ± 0.29 $n = 4$	57.0 ± 14.7 $n = 4$
HSV-1 (10^3 PFU)	60	0/6	5/6	5/6	$3.71 \pm 0.44^*$ $n = 6$	67.0 ± 22.3 $n = 6$

[1]As detected by immunohistochemistry.
[2]As detected by *in situ* hybridization.
[3]NA = not applicable.
[4]ND = not done.
$^*P < 0.05$ compared to saline.

evidence of focal chronic inflammation in brain tissues (Table 1). Inflammatory changes were less extensive than those detected in tissues derived from animals euthanized 5 to 8 days after HSV-1 inoculation but showed similar distribution in the nervous system. Inflammatory cell infiltrates were composed of mononuclear cells and involved both brain parenchyma and meninges (Figure 1C). HSV-1 protein expression was not detected by immunohistochemistry in sections of brain tissues derived from mice euthanized 60 days after inoculation (Figure 1G, Table 1). However, HSV-1 LAT expression was detected by *in situ* hybridization in the brain of five of six mice examined (Figure 2C, Table 1). LAT expression primarily involved cells with the morphologic features of neurons. Because LAT expression occurs during both productive and latent infection, and HSV-1 protein expression occurs only during productive infection (Wagner and Bloom, 1997; Roizman and Knipe, 2001), these findings indicate that productive HSV-1 infection had subsided in the brain of mice by 60 days following virus inoculation. Sections derived from brain tissue of control animals euthanized 60 days following saline inoculation demonstrated normal histology and no evidence of HSV-1 protein or LAT expression (Figures 1D, 1H, 2D, Table 1).

TUNEL analysis of brain tissues derived from mice euthanized sixty days after HSV-1 inoculation showed occasional TUNEL-positive cells that were present primarily in inflamed brain areas (Figure 3C). Morphologically, TUNEL-positive cells consisted of both neurons and nonneuronal cells. CNS tissues derived from mice euthanized 60 days following mock-infection with saline contained no TUNEL-positive cells (Figure 3D). These findings suggest that HSV-1 infection is associated with apoptosis in the murine brain several weeks after recovery from the symptomatic phase of acute encephalitis.

Brain tissue of mice euthanized 60 days after HSV-1 inoculation demonstrated significantly elevated F_2-IP levels relative to mock-infected ($P = 0.04$) and untreated controls ($P = 0.04$) (Figure 4). However, F_2-IP levels in brain tissue of mice 60 days after HSV-1 inoculation were lower than those associated with acute HSV-1 infection. Brain tissue of mice euthanized on day 60 postinoculation with HSV-1 demonstrated F_4-NP levels similar to saline-inoculated controls (Figure 5, Table 1). These findings suggest that oxidative damage to tissue may continue in the mammalian brain until at least several weeks after recovery from the symptomatic phase of HSV-1 encephalitis and that this injury primarily involves nonneuronal cells.

Discussion

In this report, we show that levels of F_2-IP and F_4-NP, which are accurate in vivo biomarkers of oxidant injury, are significantly elevated during acute HSV-1 encephalitis. In addition, we show that levels of F_2-IP remain moderately elevated in the murine brain until at least several weeks after recovery from the symptomatic phase of HSV-1 infection. Following acute infection, F_4-NP levels normalize, indicating that nonneuronal cells are the primary targets of oxidative neural injury in mice that survive acute HSV-1 encephalitis.

The demonstration by biochemical methods that HSV-1 infection is associated with oxidant brain injury confirms and extends our previous studies using immunohistochemical methods (Valyi-Nagy *et al*, 2000). As F_4-NP are highly selective, quantitative markers of neuronal oxidative damage, while F_2-IP

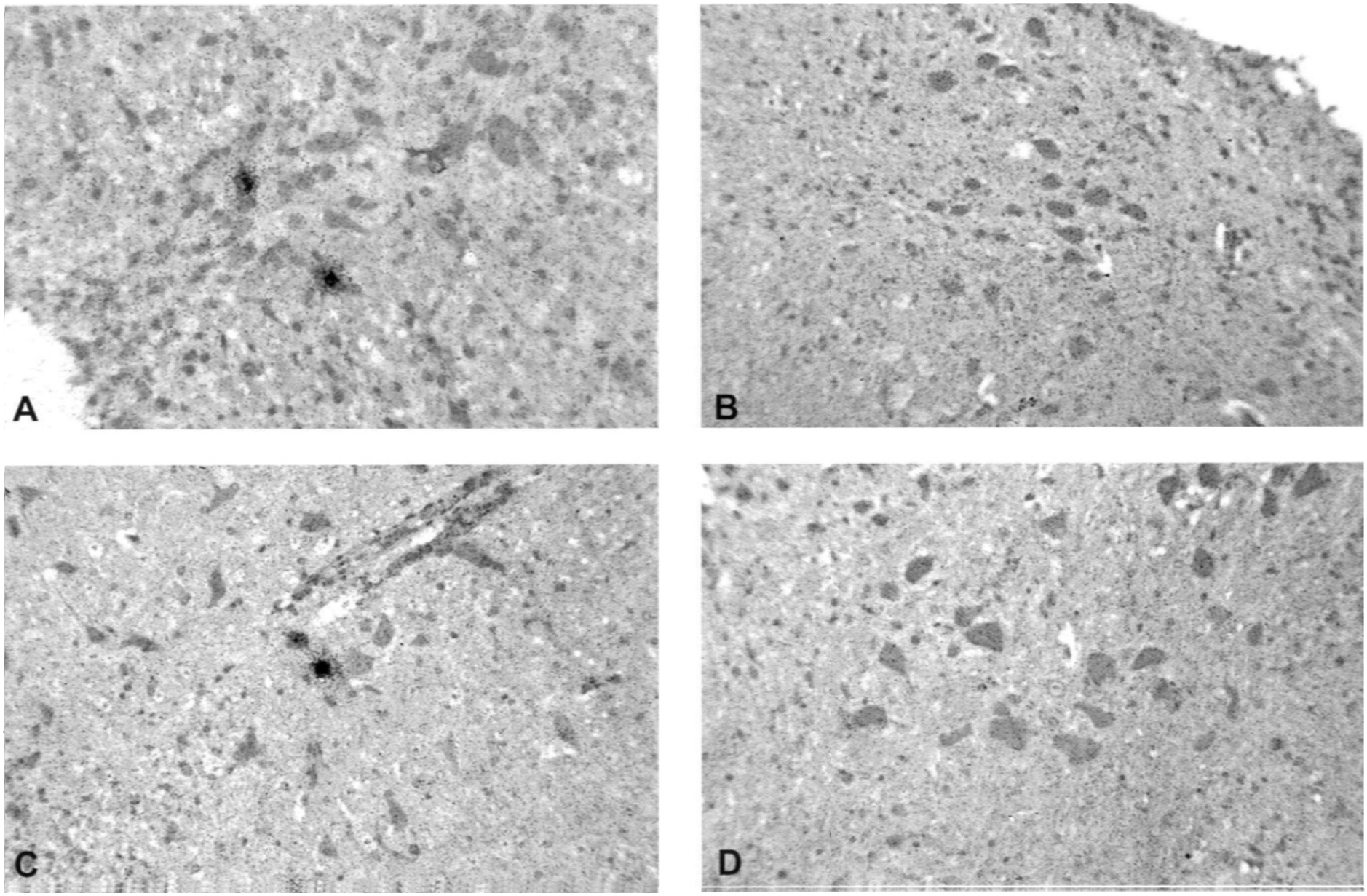

Figure 2 HSV-1 LAT expression in the right side of the brain stem of BALB/c mice following i.c. inoculation of either HSV-1 or normal saline (mock-infection) into the left cerebral hemisphere. HSV-1 LAT expression (**A**) 5 days following inoculation of 10^5 PFU of HSV-1. (**B**) 5 days following mock-infection. (**C**) 60 days following inoculation of 10^3 PFU of HSV-1. (**D**) 60 days following mock-infection. (Magnification 200×.)

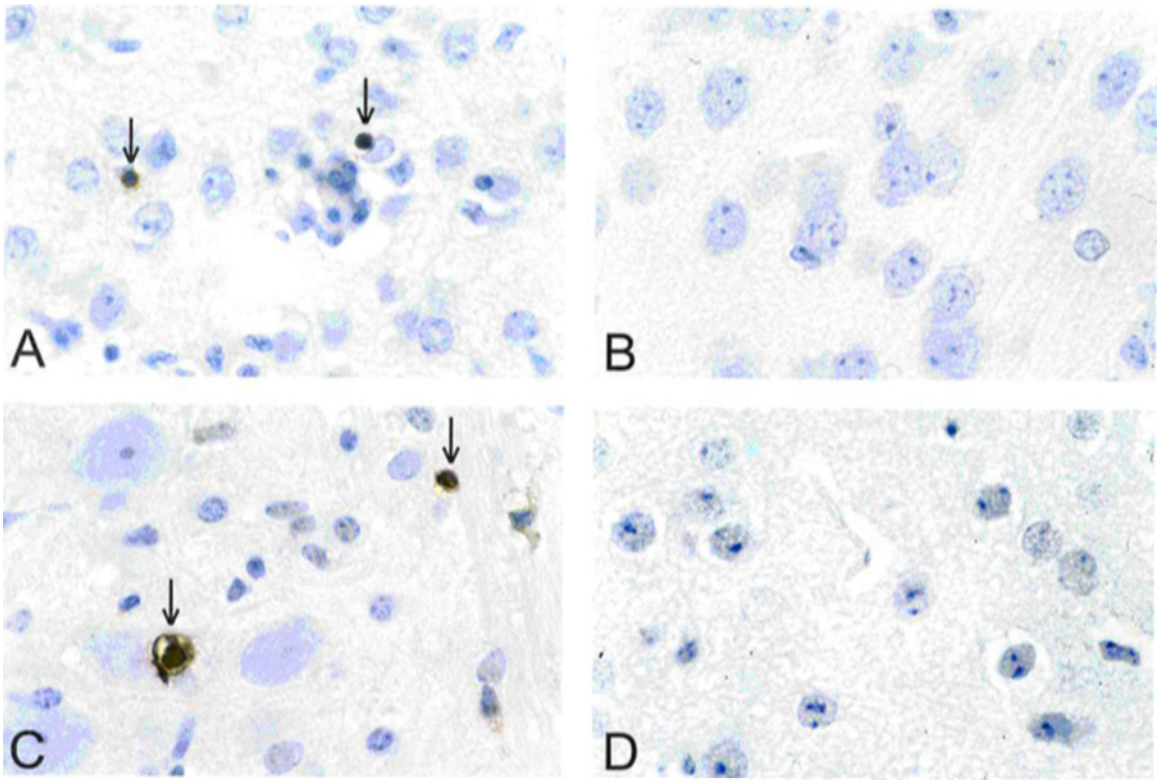

Figure 3 TUNEL-positive cells in the right cerebral hemisphere of BALB/c mice following i.c. inoculation of either HSV-1 or normal saline (mock-infection) into the left cerebral hemisphere. Sections were analyzed by TUNEL assay to detect apoptosis-associated DNA strand breaks (**A**) 5 days following inoculation of 10^5PFU of HSV-1. (**B**) 5 days following mock-infection. (**C**) 60 days following inoculation of 10^3 PFU of HSV-1. (**D**) 60 days following mock-infection. (Magnification 400×.)

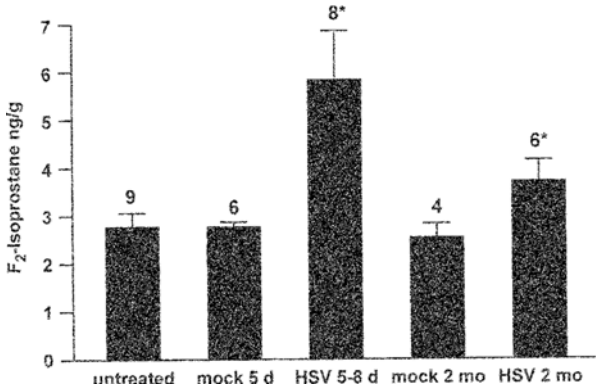

Figure 4 F_2-isoprostane (F_2-IP) concentrations in the left brain half of BALB/c mice following i.c. inoculation of either HSV-1 or normal saline (mock-infection) into the left cerebral hemisphere. Untreated: 4- to 6-week-old mice without treatment. Mock 5d: mice euthanized 5 days following mock-infection. HSV 5-8 d: six mice euthanized 5 days following inoculation of 10^5 PFU of HSV-1 and two mice following inoculation of 10^3 PFU of HSV-1. Mock 2 mo: mice euthanized 60 days after mock-infection. HSV 2 mo: mice euthanized 60 days following inoculation of 10^3 PFU of HSV-1. Numbers above the bars refer to the number of animals in the indicated group. *indicates $P < 0.05$ compared to mock-infection.

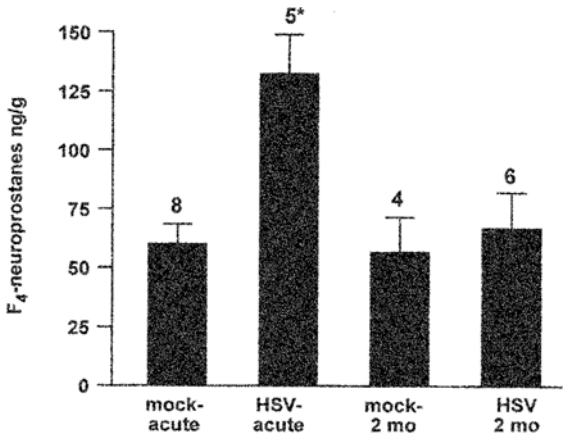

Figure 5 F_4-neuroprostane (F_4-NP) concentrations in the left brain half of BALB/c mice following i.c. inoculation of either HSV-1 or normal saline (mock-infection) into the left cerebral hemisphere. Mock acute: mice euthanized 5 days following mock-infection. HSV acute: three mice euthanized 5 days and two mice euthanized 8 days following inoculation of 10^3 PFU of HSV-1. Mock 2 mo: mice euthanized 60 days following mock-infection. HSV 2 mo: mice euthanized 60 days following inoculation of 10^3 PFU of HSV-1. Numbers above the bars refer to the number of animals in the indicated group. *indicates $P < 0.05$ compared to mock-infection.

are markers of oxidative damage to brain tissue not limited to a certain cell type, our findings indicate that oxidant injury is a mechanism of neuronal damage during acute HSV-1 encephalitis. These results do not identify specific viral or host factors responsible for oxidant brain injury during HSV-1 encephalitis. However, as HSV-1 infection has been found to cause free radical-mediated injury in cultured cells (Palu *et al*, 1994), it appears possible that both viral and inflammatory mediators are responsible for the observed effects.

Steady-state levels of free radical-mediated tissue damage represent a balance between rates of damage caused by pro-oxidant stimuli and rates of antioxidant and tissue repair mechanisms that decrease ROS and RNS levels and remove oxidatively damaged molecules (Halliwell and Gutteridge, 1999). Observations from our laboratory indicate that the turnover of F_2-IP and F_4-NP in the mammalian brain is quite rapid. In the rat hippocampus, markedly elevated F_2-IP levels associated with kainate-induced seizures return to baseline levels by 4 days after kainate injection (Patel *et al*, 2001). Furthermore, we have found that increased F_2-IP and F_4-NP levels induced by intracerebroventricular inoculation of bacterial lypopolysaccharide return to normal by 2 to 9 days after inoculation (Milatovic *et al*, unpublished observation). These findings strongly suggest that the detection of elevated F_2-IP in brain tissue of mice several weeks after recovery from symptomatic HSV-1 encephalitis is not due to residual damage from the acute phase of infection. Furthermore, the detected divergence of F_2-IP and F_4-NP levels 2 months after virus inoculation suggests that oxidant brain injury following resolution of HSV-1 encephalitis primarily affects nonneuronal cells. As we found no evidence of HSV-1 protein expression in the brain of mice 2 months after virus inoculation, it appears likely that inflammatory mediators rather than direct viral toxicity are primarily responsible for the observed effects.

HSV-1 encephalitis in the murine brain is associated with apoptosis of neurons and nonneuronal cells (Geiger *et al*, 1995, 1997; Pelosi *et al*, 1998; Valyi-Nagy *et al*, 2000). Cells undergoing apoptosis in the setting of HSV-1 encephalitis colocalize with sites of virus infection and oxidative damage in the brain (Valyi-Nagy *et al*, 2000). Findings reported here indicate that elevated F_2-IP and F_4-NP levels during acute HSV-1 infection are associated with increased apoptosis in the CNS. Furthermore, moderately elevated F_2-IP levels 2 months after HSV-1 inoculation are associated with moderately increased numbers of apoptotic cells in the murine brain. As oxidative damage can lead to cell death by apoptosis (reviewed by Halliwell and Gutteridge, 1999), these findings raise the possibility that oxidative damage is a determinant of cell death during HSV-1 encephalitis. In addition, these findings suggest that HSV-1 infection-induced oxidative damage may lead to apoptosis in the mammalian brain several weeks after recovery from the symptomatic phase of acute encephalitis.

Human survivors of HSV-1 encephalitis continue to harbor HSV-1 DNA in the brain and exhibit evidence of chronic inflammation in the absence of virus replication for months to years (Ellison and Love, 1996). It is not known whether carriage of HSV-1 DNA is associated with brain injury in survivors of encephalitis or in those much more numerous individuals also harboring HSV-1 DNA in their brain as a consequence of less severe primary infections. Because a significant proportion of the adult human population contains HSV-1 DNA in the brain, the pathological significance of this infection is important to understand. The presence of HSV-1 DNA in human brain in the context of the apolipoprotein E epsilon 4 allele has been proposed to represent a strong risk factor for the development of the most common human neurodegenerative disease, Alzheimer's disease (Itzhaki *et al*, 1997; Dobson and Itzhaki, 1999). Although an association between HSV-1 infection and Alzheimer's disease remains to be proven, our findings indicate that oxidative destruction of tissue may continue in the mammalian brain until at least several weeks after recovery from the symptomatic phase of HSV-1 infection.

Findings reported here represent to our knowledge the first quantitative biochemical demonstration of free radical-mediated tissue injury induced by a virus in the nervous system. The role of viral and host factors in the pathogenesis of virus-induced oxidant tissue injury in the nervous system is almost completely unknown. Demonstration of the feasibility of quantitative biochemical detection of free radical-mediated tissue injury induced by HSV-1 in the murine nervous system provides a new experimental system for dissection of mechanisms underlying oxidant tissue injury associated with neurotropic viral infections.

Materials and methods

Virus inoculation and tissue collection

4- to 6-week-old female BALB/c mice (Harlan, Indianapolis, IN) were inoculated by the i.c. route in the left cerebral hemisphere with 1×10^3 or 1×10^5 PFU of HSV-1 strain 17^+ or sterile normal saline (mock-infection) under anesthesia (Valyi-Nagy *et al*, 1994). The inoculum volume was 5 μL. Mice were observed three times daily for signs and symptoms of disease, and those demonstrating advanced encephalitis (lethargy, paralysis) were euthanized. Survivors of acute HSV-1 infection were euthanized 2 months after inoculation. Groups of saline-inoculated mice were euthanized 5 days and 2 months after mock-infection.

Brain tissue of euthanized mice was removed and divided into right and left halves in the midsagittal plane. Tissues derived from both sides contained

cerebrum, olfactory bulb, brain stem, and cerebellum. The left half was flash frozen in liquid nitrogen and then maintained at −80°C; the right half was fixed in 10% buffered formaldehyde. Frozen tissues corresponding to the inoculated left brain halves were used to measure F_2-IP and F_4-NP levels. Formaldehyde-fixed tissues were paraffin-embedded and sectioned. Sections were stained with hematoxylin and eosin, incubated with a polyclonal anti-HSV-1 serum, or analyzed by *in situ* hybridization for HSV-1 LAT expression.

Immunohistochemistry
Tissue sections for immunohistochemistry were deparaffinized with xylene and rehydrated through a series of graded ethanols. Endogenous peroxide was quenched in a 0.3% H_2O_2-methanol bath followed by several washes with phosphate-buffered saline (PBS). Prior to staining, binding of secondary antibodies and conjugates was blocked by appropriate reagents provided by the manufacturer. HSV-1 proteins were detected using a 1:1000 dilution of a polyclonal anti-HSV-1 antiserum raised in a rabbit (Dako, Carpinteria, CA). Tissue sections were incubated with primary antibody at 43°C for 32 min prior to addition of biotinylated anti-rabbit-immunoglobulin secondary antibody, avidin-horse-radish-peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris-HCl (pH 7.4) and 0.025% H_2O_2 as a chromogen (Ventana Medical Systems, Tucson, AZ).

***In situ* hybridization** *In situ* hybridization was performed using a nick-translated ^{35}S-labeled DNA probe specific for HSV-1 LAT (Bst2-Bst2 fragment) (Valyi-Nagy *et al*, 1992) using previously described techniques (Valyi-Nagy *et al*, 1991). The probe used in these experiments had a specific activity of $>10^8$ cpm per microgram.

Detection of apoptosis Apoptotic cell death in the brain of HSV-1-infected and mock-infected mice was determined by detection of apoptosis-associated DNA strand breaks in tissue sections using a TUNEL assay (ApopTag Plus; Onco, Gaithersburg, MD).

Quantification of F_2-isoprostanes (F_2-IP) and F_4-neuroprostanes (F_4-NP) A stable isotope dilution method followed by gas chromatography and negative ion chemical ionization mass spectrometry was used to determine F_2-IP and F_4-NP concentrations in the frozen left brain half of euthanized mice according to previously described methods (Morrow and Roberts, 1997; Roberts *et al*, 1998).

Data analysis Tissue sections processed for immunohistochemistry and *in situ* hybridization were reviewed by an observer blinded to the nature of the infection (HSV-1 or mock). Results were analyzed using the one-tailed *t*-test.

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