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Role of Nitric Oxide in Pathogenesis of Herpes Simplex Virus Encephalitis in Rats

Shigemoto Fujii, Takaaki Akaike, and Hiroshi Maeda[1]

Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan

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The role of nitric oxide (NO) in the pathogenesis of viral encephalitis was investigated by using an experimental model of herpes simplex virus type 1 (HSV-1) encephalitis in Lewis rats. The expression of inducible NO synthase (iNOS) mRNA determined by Northern blotting was observed first in the olfactory bulb and the brain stem on day 5 after intranasal inoculation of HSV-1, and thereafter iNOS mRNA was detected in other brain regions, i.e., cerebrum and cerebellum. In various parts of the brain, excessive NO production was identified by electron spin resonance spectroscopy. The temporal and spatial patterns of iNOS expression coincided with those of viral propagation, as demonstrated by polymerase chain reaction for HSV-1 gene expression as well as by the plaque-forming assay. Immunohistochemical study determined that iNOS was localized mainly in monocyte-derived macrophages. Treatment of virus-infected animals with the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA), but not N^{ω}-monomethyl-D-arginine, significantly ameliorated not only clinical symptoms such as paralysis and seizures but also mortality. Virus yield from brain tissue was not affected by L-NMMA treatment. It is of interest that increased expression of the antioxidant enzyme heme oxygenase-1 was observed in the HSV-1-infected brain; this increased expression was strongly inhibited by L-NMMA treatment. These data suggest that the high level of NO produced by iNOS is a pathogenic factor in HSV-1-induced encephalitis in rats.

INTRODUCTION

Nitric oxide (NO) produced in biological systems mediates a diverse array of physiological functions (Furchgott and Vanhoutte, 1989; Choi, 1993; Moncada and Higgs, 1993). In particular, a large amount of NO generated by the inducible isoform of nitric oxide synthase (iNOS) has been demonstrated to have a beneficial effect in host defense mechanisms against bacteria and parasites (Granger *et al.,* 1988; Nathan and Hibbs, 1991; Doi *et al.,* 1993; James, 1995; Umezawa *et al.,* 1997). However, it has also been shown that NO and its oxidized intermediates such as peroxynitrite cause cytotoxic effects and tissue damage and participate in the pathogenesis of various diseases (Beckman and Koppenol, 1996; Rubbo *et al.,* 1996).

As in bacterial and parasitic infections, iNOS expression, which brings about overproduction of NO, has been documented in various viral infections *in vivo* (Akaike *et al.,* 1998). Although the suppressive effect of NO on virus replication was reported for some viruses including coxsackievirus (Zaragoza *et al.,* 1997, 1998), Epstein–Barr virus (Mannick *et al.,* 1994), and herpes simplex virus type 1 (HSV-1) (Croen, 1993; Karupiah *et al.,* 1993; Nathan, 1997; MacLean *et al.,* 1998), it seems that excessive NO production is not necessarily beneficial for hosts experimentally infected with a number of viruses (Akaike *et al.,* 1996, 1998; Kreil and Eibl, 1996; Adler *et al.,* 1997). For example, use of NOS inhibitor to treat mice infected with influenza virus resulted in amelioration of virus-induced pneumonia, suggesting that NO produced during the infection is involved in viral pathogenesis. In addition, Adler *et al.* (1997) reported that suppression of NO biosynthesis led to improvement of HSV-1-induced pneumonia in mice despite moderate impairment of antiviral defense in the mice.

However, the biological effect of NO in viral pathogenesis is still unsettled. Furthermore, there are as yet few reports that reveal the pathogenic mechanism of HSV-1-induced encephalitis in light of the pathophysiological function of NO *in vivo.* In the present experiment, we investigated the role of NO biosynthesis in encephalitis caused by HSV-1 in rats. iNOS expression and NO production in each region of the brain were analyzed in detail during HSV-1 infection. The pathological consequence of NO overproduction was examined by assessing the effect of NOS inhibitor on clinical symptoms and mortality as well as virus growth in this experimental encephalitis. In addition, the function of NO was studied in view of heme oxygenase-1 (HO-1) induction, which appears to be critically involved in the defense mechanism against various oxidative stresses (Maines, 1997). Our present results indicate that NO may induce neuronal damage in this viral encephalitis.

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-96-362-8362. E-mail: msmaedah@gpo.kumamoto-u.ac.jp.

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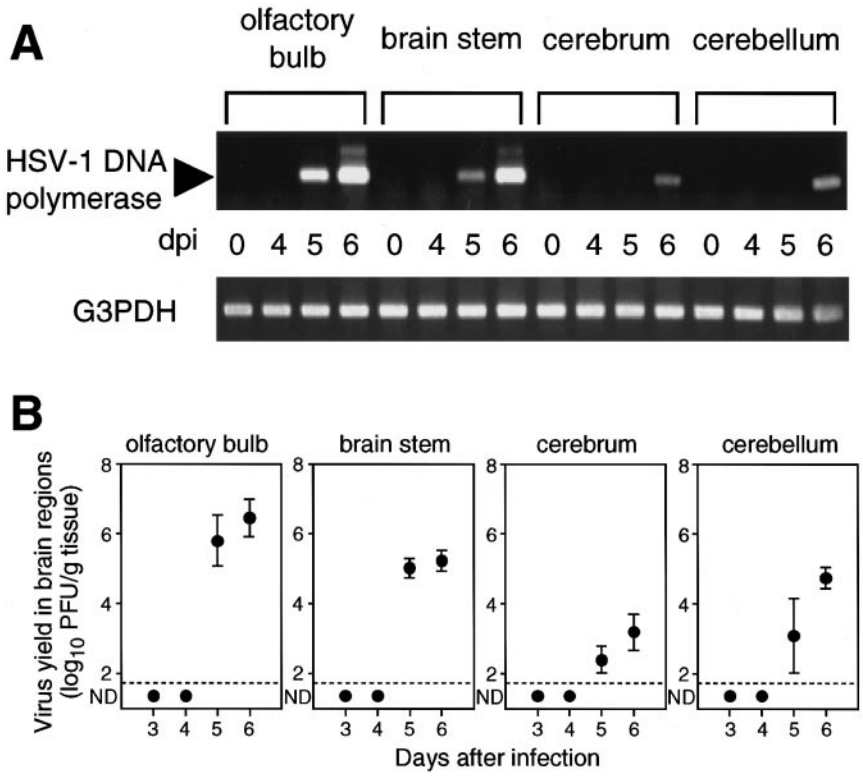

FIG. 1. Time profile of viral replication in each brain region after HSV-1 infection in rats, assessed by HSV-1 DNA polymerase mRNA expression (A) and by plaque-forming assay (B). At various time points after intranasal inoculation of 15 LD_{50} HSV-1, brains were resected and the total RNA content of each brain region extracted by the guanidine thiocyanate lysis method was subjected to RT-PCR analysis for HSV-1 polymerase mRNA expression. No apparent PCR product for HSV-1 DNA polymerase was observed until 3 days after HSV-1 infection (data not shown). Similarly, the virus yield in each brain region was quantified by using a plaque-forming assay with CV-1 cells in culture. ND, not detected. $n = 3$ for each time point and brain region. Data are means ± SEM.

RESULTS

Propagation of HSV-1 in brain tissue and production of encephalitis in rats

No apparent clinical symptom due to HSV-1 infection was observed until 3 days after the Lewis rats were inoculated with 1.0×10^5 PFU (15 50% lethal dose (LD_{50})) of virus. At 4 to 5 days after virus infection began, all animals showed not only nonspecific illness including blepharostenosis, sanguineous tears, and decreased motility but also typical clinical signs of neurological disorders, e.g., paralysis and seizures. Thereafter, the animals were highly emaciated, with considerable loss of body weight. All rats became moribund between 6 and 10 days after infection with this lethal dose of HSV-1.

The time profile of HSV-1 replication in the brain was investigated by using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for HSV-1 DNA polymerase mRNA as well as plaque-forming assay (Figs. 1A and 1B). HSV-1 DNA polymerase mRNA expression and the infectious virus were not detected in the four regions of the brain, i.e., olfactory bulb, brain stem, cerebellum, and cerebrum, until 4 days after virus inoculation. On day 5 after infection, the HSV-1 replication was initially identified in the olfactory bulb and the brain stem. Six days after the infection began, appreciable levels of virus yield were clearly seen in all brain regions. The RT-PCR analysis for HSV-1 DNA polymerase mRNA showed a similar time course of virus replication as assessed by the plaque-forming assay of the tissue homogenate obtained from each brain region.

When the rats were infected with HSV-1 at a dose of 1.0×10^4 PFU (1.5 LD_{50}), the time profile of neurological and clinical signs as well as virus replication in the brain was delayed almost 1 day during the course of the infection compared with the time profile for the larger dose, 15 LD_{50}.

Expression of iNOS mRNA in HSV-1-induced encephalitis

Expression of iNOS in the brain of rats after infection with HSV-1 at 15 LD_{50} was examined by using Northern blot analysis (Fig. 2). iNOS expression was not observed in any brain area until 4 days after infection. iNOS mRNA was first observed apparently in the olfactory bulb and brain stem on day 5 after infection; on day 6, strong iNOS induction was observed in the olfactory bulb and brain

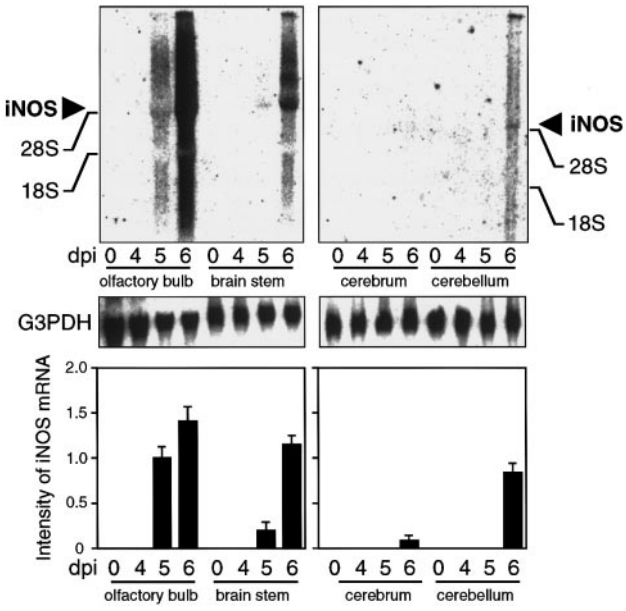

FIG. 2. iNOS mRNA expression in rat brain after HSV-1 infection. Rats were inoculated with HSV-1 (15 LD_{50}), and the total RNA content was extracted from each brain region in the same manner as described in the legend to Fig. 1. Northern blot analysis was performed by using a cDNA probe for rat iNOS to examine the time course of iNOS expression in each brain area. The level of G3PDH mRNA expression is shown as the control gene expression in the tissue. No appreciable iNOS mRNA signal was obtained until 3 days after HSV-1 infection. (Bottom) iNOS mRNA signals obtained from three different animals were quantified by densitometric analysis after visualization of the hybridized membrane with Northern blotting, using a Macintosh computer with an Image Scanner (GT6500, Epson Co., Ltd., Tokyo, Japan) and the public domain NIH Image program. Data for iNOS mRNA signal intensity are shown as means ± SEM ($n = 3$).

stem, and moderate but significant expression of iNOS was obtained in the cerebellum. The iNOS mRNA signal became evident throughout the brain on day 7 after viral infection (data not shown). The time profile of iNOS mRNA expression in the brain infected with 1.5 LD_{50} HSV-1 was similar to that for the 15 LD_{50}-infected rats except that the onset of iNOS induction in the 1.5 LD_{50} infection was delayed 1 day.

The spatial and temporal patterns of iNOS mRNA expression (Fig. 2) agreed with those of virus propagation as discussed above and shown in Fig. 1, suggesting that iNOS expression in HSV-1-infected neuronal tissue is brought about as part of the host response to virus in brain tissue.

NO production in HSV-1-induced encephalitis determined by electron spin resonance (ESR) spectroscopy

The time profile of NO production was directly measured, by using ESR spectroscopy with an NO spin trapping agent, for the brain stem and olfactory bulb (Fig. 3), where the most extensive virus invasion and iNOS mRNA expression were found (Figs. 1 and 2). NO production, as assessed by the formation of the nitrosyl adduct of *N,N*-diethyldithiocarbamate · $3H_2O$ (DETC)–Fe^{2+}, was directly identified in the brain stem and olfactory bulb of HSV-1-infected rats (Fig. 3A). No appreciable ESR signal derived from the NO–DETC–Fe adduct was observed in rat brains obtained from normal rats and those obtained within 3 days after HSV-1 infection (15

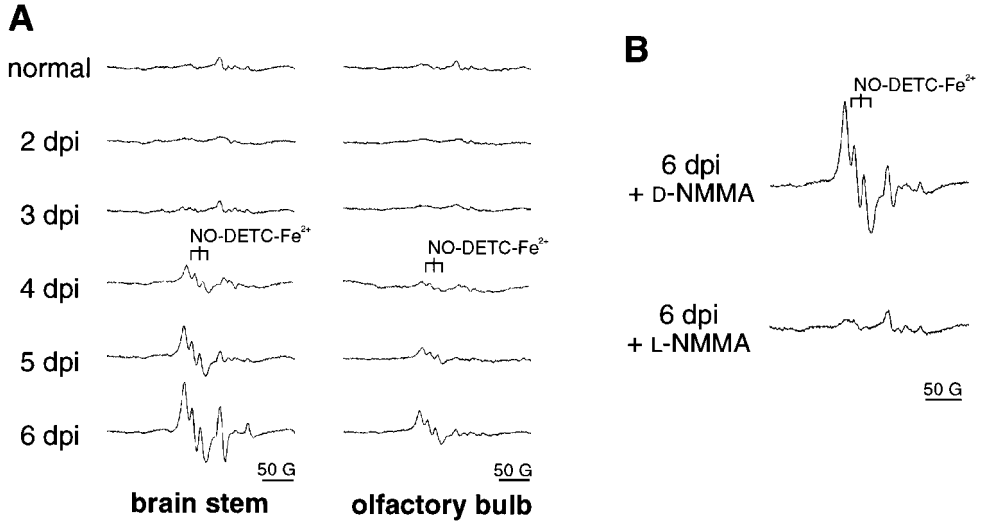

FIG. 3. Identification of NO overproduction in the HSV-1-infected brains by using ESR spectroscopy. Generation of NO was analyzed directly by ESR spectroscopy with an NO spin trap, DETC–Fe complex. The rats were infected with 15 or 1.5 LD_{50} HSV-1 in the same manner as noted in the legend to Fig. 1. (A) At each time point after HSV-1 infection (15 LD_{50}), ESR of HSV-1-infected brain was measured at 110K by using the X-band Bruker ESR spectrometer (ESP 380E) 30 min after DETC–Fe administration to the animals (see text for details). The ESR signal derived from NO–DETC–Fe adducts is seen, as verified by its typical triplet hyperfine structure. (B) Effect of D-NMMA or L-NMMA treatment on NO–DETC–Fe adduct formation. ESR study was performed in the same manner as in (A) with the brain stem of rats infected with 1.5 LD_{50} HSV-1 with or without D-NMMA or L-NMMA treatment. Two hours after the treatment of the infected animals (ip; 100 mg/kg of D-NMMA or L-NMMA), DETC–Fe was injected, followed by ESR measurement.

LD_{50}). A low level of NO production was identified as early as day 4, although the yield of HSV-1 was still below the detection limit by both plaque-forming assay and RT-PCR analysis. Also, because iNOS mRNA was not detected by Northern blotting on day 4, ESR measurement with use of a NO spin trap DETC–Fe complex seems to be more sensitive than Northern blotting analysis for iNOS expression *in vivo.* The time course of NO production later than 4 days after infection was almost parallel to that of iNOS mRNA expression and HSV-1 replication in the olfactory bulb and the brain stem (Figs. 1 and 2). It is important to note that the formation of the NO–DETC–Fe adduct was almost completely nullified by treatment of the HSV-1-infected rats (1.5 LD_{50}) with the NOS inhibitor N^{ω}-monomethyl-L-arginine monoacetate salt (L-NMMA), but not with its noninhibitory enantiomer N^{ω}-monomethyl-D-arginine (D-NMMA) (Fig. 3B). These results indicate that iNOS induced in the HSV-1-infected region is functioning biologically to produce an excessive amount of NO.

Immunohistochemical analysis for localization of iNOS-producing cells

Immunohistochemical analyses were performed to identify the cells expressing iNOS. In the brain stem section obtained 6 days after HSV-1 infection, the iNOS-positive cell as detected by anti-iNOS antibody was localized in the trigeminal nucleus (Fig. 4A). Also, by using TRPM-3, which is an antibody against a monocyte-derived macrophage (Setoguchi *et al.,* 1996), we found that there was also significant inflammatory infiltration of macrophages in the sequential section (Fig. 4B). The morphology and distribution of iNOS-positive cells were similar to those of the monocyte-derived macrophages (Figs. 4A and 4B), suggesting that the monocyte-derived macrophage was a major contributor to iNOS expression in the HSV-1-infected lesion. However, there are a few cells that expressed iNOS but did not stain with TRPM-3, indicating that iNOS expression may be attributable not only to the infiltrating macrophages but also to the microglia or astrocytes as reported previously (Bi *et al.,* 1995).

Control staining for iNOS in which the first antibody (anti-iNOS) was omitted was performed with sequential sections. No appreciable immunostaining was observed by the control staining (Fig. 4D), whereas a positive staining was evident in an adjacent section with this antibody (Fig. 4C). Similar results were obtained for TRPM-3. These data warrant unambiguously the specificity of the anti-iNOS and TRPM-3 antibodies.

Effect of NOS inhibition on HSV-1 encephalitis

To clarify the role of NO produced in HSV-1-induced encephalitis in rats, L-NMMA was administered. Treatment with L-NMMA was started at 3 days after virus infection, because excess NO production was induced later than 3 days after infection as shown in Fig. 3. The survival rate of rats infected with 1.5 LD_{50} HSV-1 was 25% (vehicle control, $n = 8$), which was markedly improved to 75% by L-NMMA treatment during the observation period of later than 10 days after infection ($n = 8$, $P < 0.05$ compared with the control group; Fig. 5). The lethality of HSV-1 infection was not affected appreciably by treatment with D-NMMA (no significant difference was found between vehicle- and D-NMMA-treated groups; $P > 0.05$).

There was no significant difference in virus yield in each brain region between control rats ($n = 6$) and L-NMMA-treated rats ($n = 5$) (Fig. 6). Although results were not statistically significant, L-NMMA administration to HSV-1-infected animals tended to reduce virus propagation in all sections of the brain. D-NMMA, however, did not show any apparent effect on HSV-1 replication in the brain (data not shown).

Expression of HO-1 mRNA in HSV-1-infected brain

HO-1 expression in HSV-1-induced encephalitis was examined as a marker for oxidative stress (Maines, 1997). The upregulation of HO-1 mRNA, as revealed by Northern blotting, paralleled the time profile of virus replication and iNOS expression, producing a high level of NO in the HSV-1-infected brain (Fig. 7). The increase in HO-1 mRNA expression was suppressed to a great extent by L-NMMA (Fig. 8), almost to the level of noninfected controls, but D-NMMA had no such suppressive effect (data not shown). This finding suggests that NO may contribute to the upregulation of HO-1, possibly through exacerbation of neuronal cell damage caused by NO produced excessively during viral infection.

DISCUSSION

In the present study, the role of NO in viral pathogenesis was explored with experimental HSV-1-induced encephalitis in rats. iNOS induction and NO overproduction were clearly demonstrated in the HSV-1-infected brain; the temporal and spatial patterns of iNOS expression were found to be consistent with those of HSV-1 in neuronal tissues. During the infection period, iNOS induction as well as virus replication in various parts of the brain appears to spread in the following order: olfactory bulb/brain stem → cerebellum → cerebrum. Because the viral yield on day 5 in the olfactory bulb was higher than that in the brain stem as assessed by both plaque-forming assay and RT-PCR, we interpret that HSV-1 may enter the central nervous system first through the nasal–olfactory bulb pathway, and it can also go from the nasal mucosa to the brain stem via the trigeminal ganglion. The latter route of spreading is described in a previous study (Beers *et al.,* 1993). The monocyte-derived macrophages appeared to be a major source of iNOS expres-

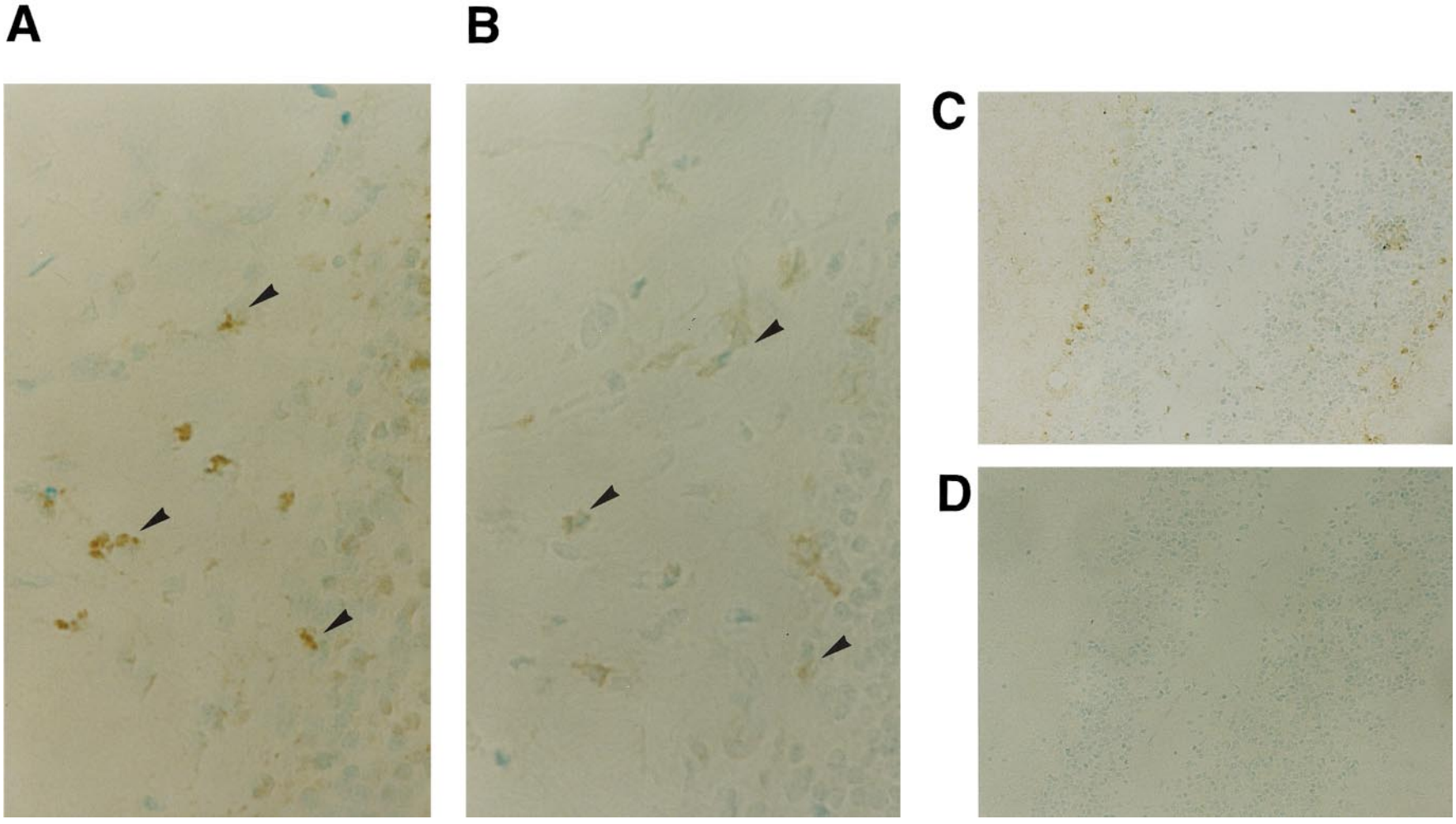

FIG. 4. Immunohistochemical staining of HSV-1-infected rat brain with a specific anti-iNOS antibody and TRPM-3. The localization of iNOS expression was examined in HSV-1-infected brain obtained 6 days after infection (15 LD_{50}) (A). With a sequential section of the infected brain shown in A, immunohistochemistry was performed with TRPM-3 antibody specific against monocyte-derived exudate macrophages (B). Immunostaining with iNOS antibody and TRPM-3 was most evident in the trigeminal nucleus of the HSV-1-infected brain, as shown (A and B). Arrows in A and B indicate localization of the cells stained with both iNOS antibody and TRPM-3. (C and D) Immunohistochemistry for iNOS was done with the sequential sections of the cerebellum, similar to (A) and (B), except that the first antibody (anti-iNOS) was omitted in (D). Magnification, ×360 (A and B) and ×90 (C and D).

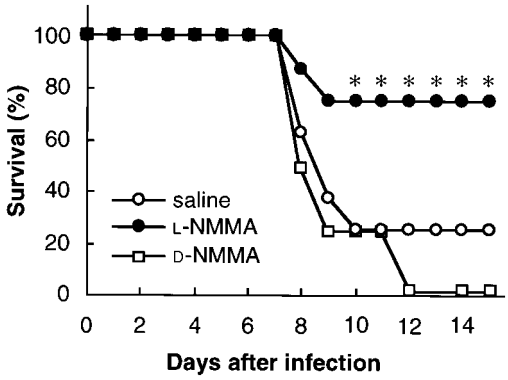

FIG. 5. The effect of L-NMMA treatment on survival rate of rats infected with HSV-1. Lewis rats were infected with 1.5 LD_{50} HSV-1, and L-NMMA (100 mg/kg body weight) was administered ip once daily from day 3 to day 7 after infection. HSV-1-infected rats given only saline served as the control group. $n = 8$ for each group; $^*P < 0.05$ versus the control group by Fisher's exact test.

sion, which was localized mainly in the inflamed area with typical pathological changes due to the viral infection. Specific NOS inhibition *in vivo* as assessed by ESR spectroscopy led to significant amelioration of encephalitis. The efficacy of NOS inhibition was substantiated by abrogation of HO-1 mRNA upregulation, which was triggered by HSV-1 intrusion into the brain, possibly through NO overproduction. These results illustrate the pathogenic effect of excessive NO generation in HSV-1-induced encephalopathy in rats.

Induction of iNOS in viral infection is now well documented for a wide range of viruses (in addition to HSV-1) that have different organ tropisms, including neuro-, pneumo-, and cardiotropic viruses such as Borna dis-

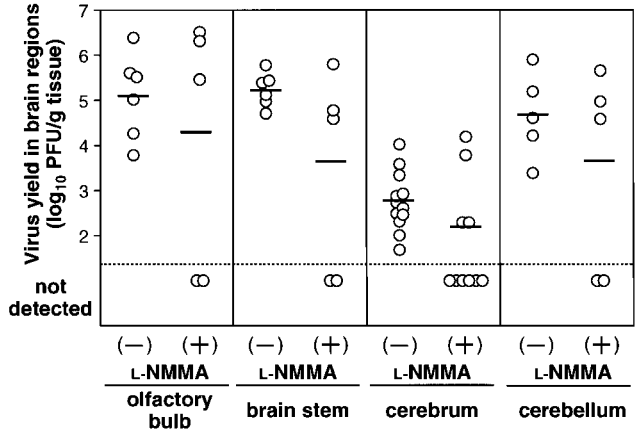

FIG. 6. Virus yield in HSV-1-infected brain of rats with or without L-NMMA treatment. HSV-1-infected rats (1.5 LD_{50}) received L-NMMA treatment (100 mg/kg body weight/day; 3 to 5 days after infection) in the same manner as described in the legend to Fig. 5. The virus yield on day 6 after HSV-1 infection was quantified by using the plaque-forming assay with CV-1 cells in culture. The horizontal bar in the figure indicates the mean value of the virus yield in each brain region ($n = 5$ for the control group; $n = 6$ for the L-NMMA-treated group).

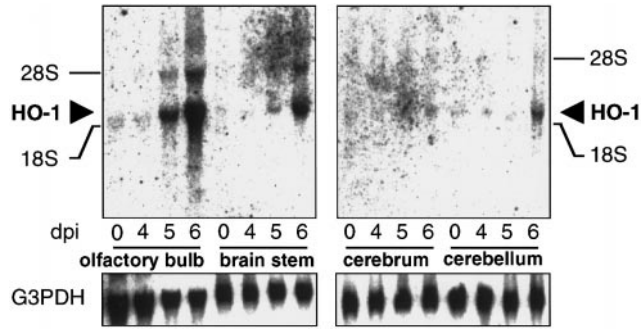

FIG. 7. Upregulation of HO-1 mRNA expression in HSV-1-infected rat brain. HSV-1-induced encephalitis was produced by the same protocol as noted in the legend to Fig. 1. HO-1 mRNA expression assessed by Northern blotting with a rat HO-1 cDNA probe in rats infected with 15 LD_{50} HSV-1.

ease virus, human immunodeficiency virus type 1, rabies virus, influenza virus, Sendai virus, and coxsackievirus (Koprowski *et al.,* 1993; Zheng *et al.,* 1993; Akaike *et al.,* 1995, 1996; Bukrinsky *et al.,* 1995; Kreil and Eibl, 1996; Mikami *et al.,* 1997; Adler *et al.,* 1997; Akaike *et al.,* unpublished observation).

NO has antimicrobial activity against bacteria, parasites, and fungi (Granger *et al.,* 1988; Nathan and Hibbs, 1991; Doi *et al.,* 1993; James, 1995; Umezawa *et al.,* 1997). Some types of virus, most typically some DNA viruses such as a murine poxvirus (ectromelia virus) and HSV-1,

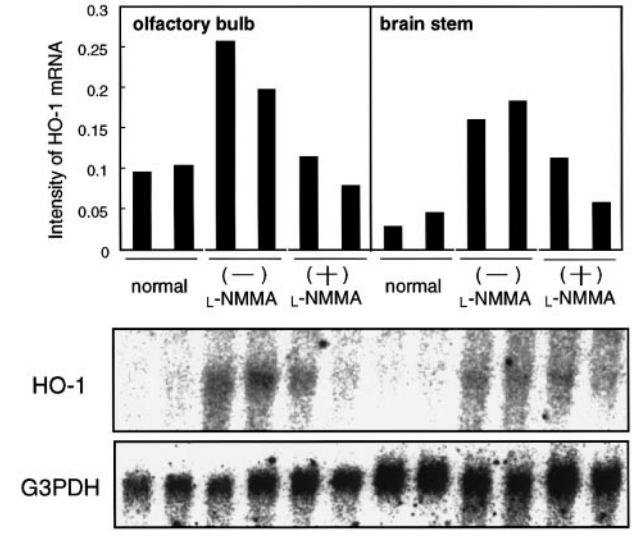

FIG. 8. The effect of L-NMMA administration on the increased level of HO-1 mRNA in rat brain regions (olfactory bulb and brain stem) 6 days after infection with 1.5 LD_{50} HSV-1. L-NMMA (100 mg/kg body weight) was given once daily (3 to 5 days after infection) to rats as mentioned in the legend to Fig. 5, and the level of HO-1 mRNA expression was determined on day 6. HO-1 mRNA signals obtained from two different animals (cf. paired bars) in each group were quantified by densitometric analysis after visualization of the hybridized membrane with Northern blotting, using a Macintosh computer with an Image Scanner.

are also susceptible to NO (Croen, 1993; Karupiah *et al.,* 1993; Nathan, 1997; MacLean *et al.,* 1998). The antiviral action of NO is not certain, however, for vaccinia virus (Rolph *et al.,* 1996) and for some RNA viruses, e.g., influenza virus and Sendai virus. Also, a discrepancy between *in vitro* and *in vivo* action has been reported in the effect of NO on coronavirus (mouse hepatitis virus) replication (Lane *et al.,* 1997).

Overproduction of NO appears to impair physiological functions of the host cells nonselectively, regardless of the direct cytopathic effect of virus replication on the host cells. Endogenous and exogenous NO is now known to be linked to mechanisms of apoptosis and necrosis in various cells including neuronal cells (Lipton *et al.,* 1993; Estévez *et al.,* 1995; Melino *et al.,* 1997). Thus, NO may be directly involved in viral pathogenesis even though it suppresses virus replication *in situ* (Adler *et al.,* 1997; Akaike *et al.,* 1998).

It is now well accepted that overproduction of NO may cause various toxic effects possibly via formation of potent cytotoxic NO-oxidized derivatives such as peroxynitrite ($ONOO^-$), which is generated by a diffusion-limited reaction of NO and superoxide (O_2^-) (Beckman and Koppenol, 1996; Rubbo *et al.,* 1996). More importantly, peroxynitrite per se is known to have proinflammatory activity, which could be exhibited through activation of cyclooxygenase (Landino *et al.,* 1996) and matrix metalloproteinases (Okamoto *et al.,* 1997). We reported previously that NO and superoxide, and their reaction product, peroynitrite, are the major pathogenic principals in a fulminant influenza virus pneumonia in mice (Akaike *et al.,* 1996). It is thus of great interest to explore the possible role of peroxynitrite in the pathogenesis of HSV-1 encephalitis in rats, in which inflammatory responses of the hosts appear to be critically involved. Such a study is now in progress in our laboratory, focusing on the mechanism of cytotoxic and proinflammatory actions of NO and peroxynitrite.

In fact, our earlier study showed that a significant improvement in survival rate was obtained with L-NMMA treatment of influenza virus-infected animals without impairment of the animals' antiviral defense (Akaike *et al.,* 1996). Similarly, Kreil and Eibl (1996) reported that NO generation does not contribute to host defense mechanisms against tick-borne encephalitis (TBE) virus, but rather it seems to enhance the pathological effect of TBE virus infection *in vivo,* as evidenced by the therapeutic effect of the NOS inhibitor aminoguanidine. Of considerable importance is a recent finding by Adler *et al.* (1997) that L-NMMA treatment led to a significant improvement of HSV-1 pneumonia as revealed by amelioration of respiratory dysfunction and pathological changes despite an increase in growth of virus in the lung. This result is consistent with our interpretation of the role of NO in the pathogenesis of HSV-1-induced encephalitis in rats.

Although an antiproliferative action of NO against HSV-1 was noted as just mentioned (Croen, 1993; Karupiah *et al.,* 1993; Adler *et al.,* 1997; Nathan, 1997; MacLean *et al.,* 1998), our present *in vivo* results indicate that L-NMMA suppression of excessive NO production in the central nervous system of HSV-1-infected animals caused improvement in neuronal disorders, but that suppression of NO generation did not influence virus propagation in the nervous tissue. The beneficial effect of NOS inhibition in HSV-1-induced encephalitis may be further supported by the result of reduced HO-1 induction after treatment of HSV-1-infected rats with L-NMMA. HO-1 is known to be induced by various stimuli, such as reactive oxygen and nitrogen species as well as hypoxia and heavy metals, so as to protect cells from oxidative injury (Kim *et al.,* 1995; Maines, 1997; Doi *et al.,* 1998). Therefore, oxidative stress caused by overproduction of NO may contribute to the pathogenesis of HSV-1-induced encephalitis. Moreover, although the effect of L-NMMA treatment on the inflammatory responses in the HSV-1 encephalitis model remains obscure, reduction of HO-1 expression by L-NMMA treatment may suggest indirectly an anti-inflammatory action of L-NMMA in HSV-1 encephalitis in rats, because HO-1 is also known to be induced by various proinflammatory cytokines (Maines, 1997).

On the basis of the present results and recent evidence in other published reports on viral pathogenesis involving NO, it is concluded that the pathological events in HSV-1-induced encephalitis seem to be closely linked not only to the direct cytopathic effect of HSV-1 but also to NO-influenced cytotoxicity mediated by the host responses.

MATERIALS AND METHODS

Virus and production of HSV-1 encephalitis

HSV-1, Fukuda strain, was used throughout the experiments (Aoki *et al.,* 1995). The virus was grown in monkey kidney epithelial cells (CV-1 cells) and was stored at −70°C until use. The experimental viral encephalitis was produced in male Lewis rats (SPF grade; 5 weeks old; 100–120 g body weight) by intranasal inoculation under light ether anesthesia at a dose of 100 μl of virus solution at 1.0×10^5 or 1.0×10^4 PFU/ml in Dulbecco's minimum essential medium (DMEM); these doses were 15 LD_{50} and 1.5 LD_{50}, respectively. All experiments were carried out according to the guidelines in the Laboratory Protocol of Animal Handling, Kumamoto University School of Medicine.

Identification of virus replication in brain tissue

Virus was quantified by use of the plaque-forming assay at various time points after HSV-1 inoculation. Rats were exsaguinated under deep anesthesia with pentobarbital sodium, and brain tissues were obtained. The rat

brain was divided into four regions (olfactory bulb, brain stem, cerebellum, and cerebrum), and each tissue was homogenized in ice-cold 10 mM sodium phosphate-buffered saline (PBS; pH 7.4) containing 0.2% bovine serum albumin. The homogenates were then centrifuged at 10,000 *g* for 10 min at 4°C, followed by filtration with a syringe filter (0.45-μm pore size; Millipore Corp., Bedford, MA). After serial dilution of the supernatant of the tissue homogenate, aliquots were inoculated onto CV-1 cell monolayers in culture. Cells were incubated for 3 days in DMEM containing 2% fetal bovine serum and 1% methylcellulose, as described previously (Aoki *et al.,* 1995), after which the number of plaques formed by virus replication was counted.

Simultaneously, HSV-1 replication in brain tissues was determined by use of RT-PCR for expression of mRNA of HSV-1 DNA polymerase. Specifically, after total RNA was extracted from the four different regions of the brain (olfactory bulb, cerebrum, cerebellum, and brain stem) using the guanidine thiocyanate lysis method with Trizol reagent (Gibco BRL, Gaithersburg, MD), 0.3 μg of the RNA was subjected to RT-PCR, which was performed according to the protocol described previously (Kimura *et al.,* 1991). The oligonucleotide primers used for HSV-1 DNA polymerase were antisense 24-mer, 5′-GGCGTAGTAGGCGGGGATGTCGCG-3′; sense 27-mer, 5′-CAGTACGGCCCCGAGTTCGTGACCGGG-3′. After an initial denaturing step at 94°C, 25 PCR cycles were performed as follows: denaturing for 45 s at 94°C, primer annealing for 90 s at 67°C, DNA synthesis for 3 min at 72°C. Similarly, mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was examined as an internal control mRNA expressed in the tissue as reported previously (Doi *et al.,* 1996). The oligonucleotide primers used for G3PDH were antisense 20-mer, 5′-CAGGGGTTTCTTACTCCTTG-3′; sense 20-mer, 5′-AAACCCATCACCATCTTCCA-3′.

Detection of iNOS mRNA expression in the brain

iNOS expression was analyzed by Northern blotting as described previously (Akaike *et al.,* 1995; Setoguchi *et al.,* 1996). Briefly, after electrophoresis of the total RNA (20 μg) extracted from the four brain regions on formaldehyde–agarose gel and transfer to a positively charged nylon membrane (Hybond-N^+, Amersham International plc, Little Chalfont, England) by a capillary transfer method, the membrane was hybridized with a DNA probe for the iNOS in 5× SSPE (0.9 M NaCl, 0.05 M sodium phosphate, and 5 mM EDTA; pH 7.7) plus 0.5% sodium dodecyl sulfate (SDS) plus 5× Denhardt's solution plus 20 μg/ml salmon sperm DNA at 65°C. Similarly, Northern blot analysis was performed with use of a cDNA fragment for G3PDH as a control housekeeping gene in the brain. The ^{32}P-labeled cDNA probe for iNOS was prepared by the random primer technique (Megaprime DNA labeling system, Amersham) using a rat iNOS cDNA fragment (−97–+429) as a template (Adachi *et al.,* 1993). After hybridization with the iNOS DNA probe, the membrane was washed twice for 10 min in 2× SSPE plus 0.1% SDS at room temperature and twice for 20 min in 0.1× SSPE plus 0.1% SDS at 65°C. The radioactive band on the hybridized membrane was detected with a Bioimage analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan).

Immunohistochemistry for iNOS expression

iNOS expression in rat brain was localized by immunohistochemical study using a specific polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for rat iNOS as reported previously (Setoguchi *et al.,* 1996). The brain was obtained as follows. At 6 days after HSV-1 infection (with the 15 LD_{50} dose), the brain was perfused with 0.0375 M sodium phosphate buffer (pH 6.2) containing 0.01 M periodate, 0.075 M lysine, and 2% paraformaldehyde (PLP solution), and the brain was then removed and immersed in PLP solution for 1 h at 4°C, followed by dehydration in sequential concentrations of sucrose. Frozen sections 6 μm thick were used for immunological detection by the peroxidase–diaminobenzidine method. After inhibition of endogenous peroxidase activity, each section was incubated with the anti-rat iNOS polyclonal antibody for 1 h at room temperature. Sections were washed with PBS (pH 7.4), after which they were incubated for 1 h with a sheep anti-rabbit immunoglobulin [$F(ab')_2$] conjugated with peroxidase diluted 1:100. Tissue-bound peroxidase activity was visualized by using 3,3′-diaminobenzidine as a substrate; methyl green was used for nuclear staining. Similarly, the localization of exudate or monocyte-derived macrophages was identified immunohistochemically by using a specific monoclonal antibody, TRPM-3 (Setoguchi *et al.,* 1996).

Identification of NO production in the brain by ESR spectroscopy

NO generation in HSV-1-infected brain was measured directly by ESR spectroscopy with a dithiocarbamate–iron complex as a spin trap for NO (Akaike *et al.,* 1996). Briefly, DETC (Wako Pure Chemical, Osaka, Japan) and $FeSO_4 \cdot 7H_2O$ were administered intramuscularly at different sites in the lower limbs of the rats at 400 and 20 mg/kg body weight, respectively. Thirty minutes after injection of DETC and $FeSO_4$, brains were perfused with saline containing heparin (10 U/ml) for 5 min under pentobarbital anesthesia, and brains were frozen immediately in a quartz sample tube. ESR spectroscopy was carried out by using an X-band ESR spectrometer (Bruker ESP 380E; Rheinsteten, Germany) at 110K. The conditions for ESR measurement were microwave power 4 mW and modulation amplitude 0.5 mT, and the magnetic field was calibrated by using TCNQ-Li salt ($g = 2.00252$).

Treatment of virus-infected rats with L-NMMA

To examine the effect of NOS inhibition on HSV-1-induced encephalitis, N^{ω}-monomethyl-L-arginine monoacetate salt (Calbiochem–Novabiochem International, San Diego, CA) dissolved in saline (0.5 ml) was injected intraperitoneally (ip) into HSV-1-infected rats at a dose of 100 mg/kg body weight/day from 3 to 7 days after viral inoculation. An enantiomer of L-NMMA, i.e., D-NMMA (A. G. Scientific, San Diego, CA), which is unable to inhibit NOS, was administered to virus-infected rats in the same manner as was L-NMMA. HSV-1-infected rats given only vehicle (saline) served as a control group.

Neurological symptoms and survival rate of HSV-1-infected animals were scored twice a day. The amounts of infectious virus in rat brain tissue with or without L-NMMA treatment were determined by use of the plaque-forming assay at 6 days after infection.

Furthermore, the efficacy of NOS inhibition by L-NMMA treatment was confirmed by ESR spectroscopy with the DETC–Fe complex as described above. Specifically, NO generation in brain tissue of HSV-1-infected rats was examined 2 h after ip injection of L-NMMA (100 mg/kg).

Expression of HO-1 in HSV-1-infected brain

It was recently reported that NO induces upregulation of HO-1, which is involved in heme degradation yielding biliverdin, which exerts potent antioxidant activity under various oxidative stresses in biological systems (Maines, 1997). Therefore, the effect of NOS inhibition was investigated by analyzing the level of HO-1 mRNA expressed in the brain during HSV-1 infection with or without L-NMMA treatment. HO-1 mRNA expression was assessed by Northern blotting with use of a rat HO-1 cDNA fragment (882 bp), which was kindly provided by Dr. Shigeki Shibahara, Tohoku University School of Medicine, Sendai, Japan (Takeda *et al.,* 1994). Radiolabeling of the HO-1 cDNA probe and hybridization were done as described above for the iNOS Northern blotting protocol.

Statistical analysis

Statistical difference was determined by the two-tailed unpaired *t* test or Fisher's exact probability test. A P value of < 0.05 was considered statistically significant.

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