Microglia are the major cellular source of inducible nitric oxide synthase during experimental herpes encephalitis

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Although production of reactive nitrogen and reactive oxygen species (RNS and ROS) is a component of innate defense against viral infection, their overproduction in the brain may also lead to deleterious consequences. To investigate potential immunopathologic roles of oxidative stress during herpes encephalitis, the authors examined the expression kinetics of inducible nitric oxide synthase (iNOS) as well as heme oxygenase-1 (HO-1), a marker of oxidative stress, and evaluated infection-induced oxidative brain damage. Results from these studies showed that both iNOS and HO-1 gene expression were highly elevated in the brain within 7 days post infection (d.p.i.) and remained elevated through 21 d.p.i. Real-time bioluminescence imaging of HO-1 promoter–luciferase transgenic mice confirmed HO-1 promoter activity in the brains of HSV-1-infected animals within 3 d.p.i., which peaked between 5 and 7 d.p.i. Immunohistochemical staining for both 3-nitrotyrosine and 8-hydroxydeoxyguanosine (8-OH-dG), as well as quantitative assessment of 8-isoprostane levels, demonstrated the presence of viral infection-induced oxidative brain damage. In addition, when brain leukocytes obtained from animals with experimental herpes encephalitis were sorted using fluorescence-activated cell sorting (FACS) and the individual cell populations analyzed, CD45(int)/CD11b(+) resident microglia were found to be the major cellular source of iNOS expression. Journal of NeuroVirology (2008) 14, 229–238.

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Introduction

Previous studies from our laboratory have shown that vigorous proinflammatory immune responses occur in the brains of herpes simplex virus (HSV)-infected BALB/c mice (Marques et al, 2006). The kinetics of these neuroimmune responses, taken together with the kinetics of mortality, suggest that early events in the course of infection play a pivotal role in the progression of experimental herpes encephalitis. We have also previously identified microglial cells as key players in orchestrating these neuroimmune responses (Marques et al, 2004). Microglia are a potent cellular source of proinflammatory cytokines, chemokines, and oxidative enzymes in the brain. The production of these innate immune mediators implies both protective as well as potentially detrimental roles during viral brain infection.

Regarded as a critical component of the innate immune response, nitric oxide (NO) has been shown to mediate an array of physiological functions. It is produced endogenously in various cell types by one of three nitric oxide synthases (NOSs; neuronal NOS, endothelial NOS, and inducible NOS [iNOS]). Neurons, endothelial cells, and macrophages are the best characterized producers of NO (Croen, 1993). NO is
known to modulate neuronal function, regulate vaso-
motor tone, and more pertinent to this study, mediate
antimicrobial activity, inflammatory responses, and
cytotoxicity (Breit and Snyder, 1992; Garthwaite,

The role of NO during the course of viral brain
infection is still unclear. Although NO has been
shown to inhibit viral replication, overproduction
of this reactive species has also been shown to in-
duce cellular apoptosis and promote tissue damage.
Evidence suggests that localized production of NO
early during HSV infection may be responsible for
decreased pathogenesis (Croen, 1993; Gamba et al,
2004; MacLean et al, 1998). In contrast, several stud-
ies also suggest a pathological role for NO during
acute HSV infections. In two different models of
HSV-induced disease, it has been reported that treat-
ment of infected animals with the non-selective NOS
inhibitor N\textsuperscript{\textsuperscript{o}}-monomethyl-L-arginine (L-NMMA) re-
sulted in significantly improved survival rates, de-
spite no decrease in viral titers (Adler et al, 1997;
Fujii et al, 1999). Further studies indicate that NO
induces proinflammatory responses by stimulating
vasodilatation and changes in vascular permeability,
which modify leukocyte adhesion and thereby alter
immune cell trafficking (Adler et al, 1997; Martin
et al, 1997). Based on such findings, it is likely that
brain damage resulting from HSV infection may be
partially attributed to host-mediated defense mecha-
nisms, specifically the release of reactive species.

In light of conflicting evidence reported regarding
the role of NO during HSV brain infection, it appears
that the reported discrepancies may be mediated
by alterations in the physiological balance neces-
sary to keep this immune defense mediator from
producing deleterious consequences. The immune
system has developed mechanisms that regulate the
overproduction of NO. The most impressive negative
feedback regulator for NO production is the antiox-
idant enzyme heme oxygenase (HO)-1 (Turcanu
et al, 1998a, 1998b). Under inflammatory conditions,
HO-1 is the rate-limiting enzyme in the degradation
of cellular heme, producing equimolar amounts of
iron, carbon monoxide (CO), and biliverdin. This cy-
toprotective anti-inflammatory stress response gene
is well known as a marker for oxidative stress and is
induced in most cell types by various stimuli such as
proinflammatory cytokines, hypoxia, heavy metals,
and, most notably, by reactive nitrogen species (RNS)
and reactive oxygen species (ROS). Of particular
relevance to this study, HO-1 is induced by NO and
this induction is also known to inhibit subsequent
NO production. The induction of HO-1 mRNA has
previously been used as a marker of oxidative stress
during HSV-1–induced encephalitis in rat brains fol-
lowing intranasal infection (Fujii et al, 1999). Taken
together with mounting evidence that shows HO-1 is
a potent mediator of both anti-inflammatory and
anti-apoptotic effects, it is plausible that this enzyme
plays an important role in protecting NO-secreting
cells and the surrounding tissue from oxidative
damage (Baranano and Snyder, 2001; Chora et al,
the intimate relationship between NO and HO-1,
we set out to investigate the expression of these
mediators in murine brains during experimental
herpes encephalitis.

**Results**

**iNOS mRNA induction during herpes encephalitis**

We first investigated the kinetics of iNOS mRNA in-
duction in the brains of animals with herpes en-
cephalitis. In these experiments, susceptible BALB/c
mice were infected via the intranasal (i.n.) route
and the infected brains were collected at 1, 7, 14,
and 21 days post infection (d.p.i.). Upon harvest-
ing, the brains were divided into three regions: cor-
tex, subcortex, and cerebellum. Total RNA was ex-
tracted from each brain region, DNase-treated, and
reverse-transcribed. The cDNA obtained was ana-
lyzed by real-time polymerase chain reaction (PCR)
using iNOS-specific primers (Figure 1). RNA lev-
els were normalized to hypoxanthine guanine
phosphoribosyl transferase (HPRT)-1 and fold induction
was calculated by comparison to uninfected controls.
Data obtained from these experiments indicate that
iNOS mRNA expression was elevated in the subcor-
tex and cerebellum of HSV-infected mice by 7 d.p.i.
and remained elevated at 21 and 14 d.p.i., respec-
tively. Interestingly, although iNOS levels in the cor-
tex peaked at a later time point (i.e., 14 d.p.i.), in-
creased expression was still detected at 21 d.p.i. in
this region.

**Kinetics of HO-1 mRNA induction**

Because HO-1 induction is a widely used indica-
tor of oxidative stress, we next investigated the ki-
etics of HO-1 mRNA expression throughout the
course of viral brain infection. HO-1 mRNA expres-
sion levels were quantified at 1, 7, 14, and 21 d.p.i.
For these experiments, cortex, subcortex, and cere-
bellum were harvested and total RNA was isolated
and reverse-transcribed. cDNA obtained was ana-
lyzed by real-time PCR using primers specific for
HO-1 (Figure 2). Transcript levels were normalized
to HPRT and data are presented as fold induction
over uninfected controls. Results obtained during
these experiments show that, similar to the kinetics
of iNOS expression, HO-1 mRNA levels were highly
elevated in the subcortex and cerebellum of mice as
early as 7 d.p.i. Levels in the cortex were also highest
at day 14 p.i. To further confirm that the HO-1 gene
was induced in the HSV-infected brain, biolumines-
cence imaging studies were performed using HSV-
infected HO-1 promoter–luciferase transgenic mice.
For these experiments, the transgenic mice were in-
fected via the i.n. route and subsequently imaged at
3, 5, 7, and 9 d.p.i. (Figure 3A and B). Baseline lev-
els of expression were also determined prior to in-
fecion. Data obtained from these experiments show