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In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases

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ABSTRACT **The purpose of this study was to investigate the induction of inducible nitric oxide synthase (iNOS) mRNA in the brain tissue of rats and mice under the following experimental conditions: in rats infected with borna disease virus and rabies virus, in mice infected with herpes simplex virus, and in rats after the induction of experimental allergic encephalitis. The results showed that iNOS mRNA, normally nondetectable in the brain, was present in animals after viral infection or after induction of experimental allergic encephalitis. The induction of iNOS mRNA coincided with the severity of clinical signs and in some cases with the presence of inflammatory cells in the brain. The results indicate that nitric oxide produced by cells induced by iNOS may be the toxic factor accounting for cell damage and this may open the door to approaches to the study of the pathogenesis of neurological diseases.**

The mechanisms involved in the development of central nervous system (CNS) lesions are readily understood only in those pathological conditions in which there is evidence that a virus destroys its target cell as a direct cytopathic consequence of viral replication (e.g., polio virus or other neurotrophic viruses; refs. 1 and 2). However, the effector mechanisms involved in tissue damage associated with a far wider variety of viral infections of the CNS, involving such viruses as the measles and rubella viruses as well as human immunodeficiency virus 1, are unclear. Likewise, the mediators responsible for the CNS damage associated with chronic neurologic diseases such as multiple sclerosis remain the subject of speculation. Interestingly, morphologic analyses have revealed that lesions in affected brain tissues are frequently surrounded by infiltrating inflammatory cell populations. Although the precise role that these cells play in CNS pathology is the subject of ongoing investigation, previous studies have focused on the ability of leukocyte populations to generate proinflammatory cytokines (e.g., interleukin 1, tumor necrosis factor, etc.), neurotoxins (e.g., quinolinic acid), or reactive oxygen intermediates (3). Recently, increased attention has focused on the possibility that reactive nitrogen intermediates (NOI) generated by a family of cytochrome P-450 reductase-like enzymes, the nitric oxide synthases (NOS), directly damage host tissues in a diverse array of pathogenic states (4).

To date, at least three NOS genes have been cloned and characterized, and these have been provisionally categorized on the basis of their sensitivity to regulation by Ca^{2+} transients (4). In this schema, NOS forms that bind calmodulin in a reversible Ca^{2+}-dependent manner are termed the constitutive forms of NOS, and those forms of the enzyme that bind calmodulin tightly at resting $[Ca^{2+}]$ are termed inducible NOSs (iNOSs). After the addition of rapid-acting agonists, the constitutive NOS system generates only low levels of the NOI, nitric oxide (NO), whereas the iNOS system begins to generate NO several hours after exposure to cytokines or microbial products (4). Because (*i*) the iNOS system can generate large quantities of NO over an extended time frame and (*ii*) NO is known to exert numerous toxic effects against a wide variety of mammalian cell targets (4), we considered the possibility that CNS tissue damage of either viral or immune origin might be associated with enhanced expression of iNOS. To this end, reverse transcription (RT)-PCR was employed to monitor the possible expression of rodent iNOS in brain tissues after infection with herpes simplex virus type 1 (HSV-1), borna disease virus (BDV), or rabies virus, as well as after the induction of experimental allergic encephalitis (EAE). Our results show that iNOS expression is rapidly induced in the CNS tissues of animals after viral- or immune-mediated insults. Because levels of iNOS mRNA expression appear to correlate with clinical severity, these results suggest that iNOS-derived NO could play an important role in the CNS damage associated with these disease states.

MATERIALS AND METHODS

Infection of Rats with BDV. Six-week-old female Lewis rats were anesthetized with methoxyfluorane (Metofane, Pitman–Moore, Mundelein, IL) and infected intranasally with 30 μl of a 10% (wt/vol) rat brain suspension containing 3×10^2 focus-forming units to 3×10^4 of BDV. At different times post-infection (p.i.), rats were euthanized by methoxyfluorane inhalation and the brains were collected.

Infection of Mice with HSV-1. Female BALB/c mice (cByJ, The Jackson Laboratory), 4–6 weeks old, were anesthetized and inoculated with 10^6 to 10^7 plaque-forming units of HSV-1 in each eye after corneal scarification, as described (5). Animals were then euthanized at the times indicated.

Infection of Mice with Rabies Virus. Female BALB/c mice (cBYJ, The Jackson Laboratory), 4–6 weeks old, were inoculated in the masseter muscle with 50 μl of a 10% suckling mouse brain suspension containing 10^3 LD_{50} units of street rabies virus isolated from a Thailand dog. At the onset of neurological signs 5–12 days p.i., animals were euthanized and brains were collected.

Induction of EAE in Rats. Female Lewis rats, 8 weeks of age and obtained from Charles River Breeding Laboratories, were injected in the rear footpad with guinea pig myelin basic protein (50 μg per animal) emulsified in complete Freund's adjuvant containing 200 μg of *Mycobacterium tuberculosis*

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Abbreviations: CNS, central nervous system; HSV-1, herpes simplex virus type 1; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; EAE, experimental allergic encephalitis; NOI, nitrogen intermediates; RT, reverse transcription; BDV, borna disease virus; p.i., post-infection.

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(H37Ra) as described (6). Animals were then euthanized at the times indicated.

RT-PCR and Southern Blot Analysis of iNOS mRNA in the Brain. RNA was extracted from fresh rat or mouse brains as described (7). RT-PCR and Southern blot analysis were performed as described (8, 9) using primers selected from the iNOS cDNA (10) as follows: iNOS antisense 30-mer, 5'-GTCGACGAGCCTCGTGGCTTTGGGCTCCTC-3'; iNOS sense 30-mer, 5'-GTCGACCTTCCGAAGTTTCTGGCAGCAGCG-3'; 30-mer hybridization probe, 5'-ACGTTCAGGACATCCTGCAAAAGCAGCTGG-3'.

Histopathology. Brains were fixed in 10% (vol/vol) buffered formalin. The tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopy.

RESULTS

As shown in Table 1, iNOS mRNA was expressed in brain tissue of rats or mice infected with BDV, HSV-1, or rabies virus as well as in rats with EAE.

iNOS in HSV-1-Infected Mice. A total of nine mice were infected via the intraocular route with HSV-1. Subsequently, individual groups (three mice per group) were euthanized on days 3, 5, and 6, and the brains were processed for histology and iNOS mRNA expression. Three days p.i., inoculated mice displayed no clinical signs of disease and iNOS mRNA could not be detected in either treated (Fig. 1) or control (data not shown) animals. However, on either day 5 or 6 p.i., all animals showed clinical signs of encephalitis and a marked induction of iNOS message in six of six animals was detected (Fig. 1 and Table 1). Interestingly, histologic analyses revealed that only three of six animals developed an acute inflammatory reaction (Table 1). Thus, although HSV-1 infection clearly induces iNOS expression, message onset does not necessarily correlate with the development of a discernible inflammatory infiltrate.

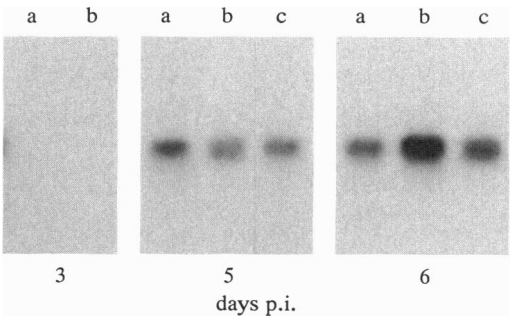

FIG. 1. Appearance of iNOS mRNA in mouse brain after intraocular inoculation of HSV-1. Mice were infected with 10^7 plaque-forming units of HSV-1, and on different days after infection, total RNA was extracted from the cerebrum. RNA extraction and RT-PCR were performed. The results displayed represent RT-PCR products from two rats (a and b) at day 3 p.i., three rats (a, b, and c) at day 5, and three rats (a, b, and c) at day 6 p.i.

Induction of iNOS Expression After BDV Infection. Rats infected with a high dose of virus (3×10^4 plaque-forming units) were euthanized at 7, 14, 26, 38, and 60 days p.i. After a 7- or 14-day incubation period, rats showed no signs of clinical illness and only a mild mononuclear infiltrate could be detected 14 days p.i. (Table 1). However, whereas iNOS message could not be detected 7 days p.i., a low level iNOS expression was observed at the latter time point (Fig. 2 and Table 1). On day 26 p.i., rats displayed severe neurological symptoms characterized histologically by generalized perivascular necrosis. Interestingly, the highest levels of iNOS message were detected by RT-PCR analysis at this time. Although this technique yields qualitative rather than quantitative results, high levels of iNOS mRNA expression were again detected in a second group of two animals that developed signs of severe neurologic damage 21 days p.i. (data not shown). However, the induction of iNOS mRNA did not necessarily correlate with symptomatology, since rats

Table 1. Presence of iNOS mRNA in brains of rats or mice with EAE and virus-induced encephalomyelitis

Disease	Days after immunization or infection	Animals, no.	Clinical sign(s)	Animals with presence of iNOS in brain tissue, no.	Animals with histological changes, no.	Nature of histological lesions
Acute HSV-1 encephalomyelitis (mice)	3	3	None	0	0	ND
	5	3	Uncoordinated, rough fur	3	1	Acute inflammation in brain stem; other brain regions had no significant lesions
	6	3	Uncoordinated, rough fur	3	2	Brain stem meningitis, acute inflammatory focus in olfactory lobe
Borna disease (rats)	7	1	None	0	0	No lesions
	14	1	None	1	1	Mild mononuclear infiltrates in the meninges
	26	1	Paralysis, convulsion	1	1	Generalized perivascular cuffing and necrosis
	38	1	Apathetic behavior	1	1	Neuronal loss in piriform cortex and dendate gyrus
	60	1	Apathetic behavior	0	1	Mild diffuse infiltration of cortex and hippocampus with mononuclear cells
Rabies (street) (mice)	10–12	7	Severe paralysis	3	7	Mild infiltration of cortex and white matter with mononuclear cells
EAE (rats)	5	2	None	1	0	No significant lesions
	9	2	None	2	0	No significant lesions
	13	2	Hind limb paralysis	2	2	Perivascular cuffs and local inflammation in optic tract, cerebral brain stem inflammation
	19	1	Recovered from paralysis	1	1	Prominent brain stem inflammation with perivascular cuffs and glial nodules

ND, none detected.

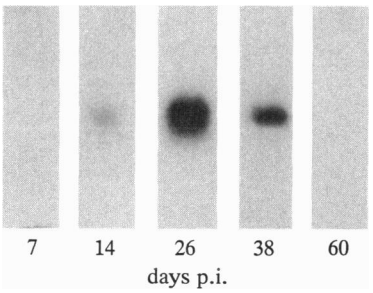

FIG. 2. Appearance of iNOS mRNA in rat brain after intranasal inoculation of BDV. Rats were infected with 3×10^4 focus-forming units of BDV. At different days after infection, total RNA was extracted from the cerebrum. The results displayed represent RT-PCR products obtained from one rat per time point. Total RNA (1 μg) was subjected to RT-PCR, and the amplified BDV-specific cDNA was analyzed.

infected with a low dose of virus (3×10^2 plaque-forming units) appeared clinically normal at 96 days p.i. while continuing to express iNOS message (data not shown). Finally, rats infected with a high dose of virus recovered from the acute disease by 38 days p.i. (Table 1). Although iNOS message could still be detected at 26 or 38 days p.i., no signal could be detected at day 60 despite the continued presence of mild histopathological lesions (Table 1 and Fig. 2).

iNOS Expression of Rabies. Of seven mice infected with a street rabies virus isolate, all were paralyzed by 10–20 days p.i. In contrast to the lesions observed in HSV-1- or BDV-induced encephalomyelitis, histologic analysis of the rabies-infected animals revealed classic minimal inflammatory reaction (Table 1 and Fig. 3). Interestingly, despite the severity of the clinical symptoms, iNOS message could only be detected in three of the seven animals.

Induction of iNOS Expression in an Animal Model of EAE. To determine whether immune-mediated CNS damage could likewise induce iNOS, EAE was induced in rats after the administration of guinea pig myelin basic protein. Tissues were then analyzed on days 5, 9, 13, and 19 p.i. (Table 1). Although neither clinical nor histologic signs developed until day 13, iNOS mRNA could be detected at significant levels in brains of animals euthanized—in one of two animals on day 5 and in two of two animals on days 9 and 13, respectively (Fig. 4). By day 19 p.i., animals recovered from their paralytic state, but histologic abnormalities and increased levels of iNOS remained evident (Table 1 and Fig. 4). Thus, in EAE, the induction of iNOS mRNA preceded detectable signs and symptoms.

DISCUSSION

This study has demonstrated that iNOS mRNA, which is not detectable in normal brain tissue, is up-regulated in the brains of rodents infected with HSV-1, BDV, or rabies virus, as well as in animals with an EAE. In all of these disease entities, iNOS message was either detected before, or coincident with, the onset of clinical symptoms. Although RT-PCR analysis is not qualitative in the absence of appropriate internal standards, the levels of message detected appeared to correlate with the severity of clinical signs, and in some cases, with the extent of the inflammatory reaction in the brain. Interestingly, in BDV-infected rats, the levels of iNOS mRNA not only correlated with the degree of neurological involvement and CNS inflammation but also with the levels of tumor necrosis factor α, interleukin 1α, and interleukin 6 mRNAs (11), which could in turn act as potential mediators of iNOS expression (4). Indeed, in a manner similar to that observed with cytokine messages, iNOS mRNA appeared to peak at 26 days p.i. when neurologic damage was most severe (11). This observation supports the contention that certain cytokines, such as tumor necrosis factor α and interleukin 1α, may participate in the inflammatory process by triggering infiltrating macrophages to generate NOI. However, given the fact that iNOS expression increased under conditions in which only a mild inflammatory reaction was provoked (e.g., early BDV infection and rabies), it seems likely that the other cellular sources of NOI operate in this milieu. Indeed, Lowenstein *et al.* (12) have reported that endotoxin can trigger the rapid induction of iNOS in rat brain *in vivo*, and Galea *et al.* (13) demonstrated that astrocytes and glial cells express iNOS activity *in vitro*.

In addition to the introduction of iNOS observed after viral insults, we have also demonstrated that iNOS expression increases in a model of CNS autoimmune disease. Recently, MacMickling *et al.* (18) demonstrated that leukocytes recovered from the peripheral blood or CNS in an animal model of hyperacute experimental autoimmune encephalomyelitis are induced to generate increased quantities of NOI and reactive oxygen intermediates as a result of exposure to soluble factors produced by autoreactive T cells (14). In our study, brain-associated iNOS was detected by day 5 p.i., at least 4 days before any pathological changes were seen in the brain. Presumably, small numbers of activated T cells homed to the CNS (15) soon after priming in the periphery and subsequently generated cytokines, which induced iNOS expression either in infiltrating macrophages or resident endothelial cells, astrocytes, or glial cells (4, 13, 16). Alternatively, it is possible that responding cell populations were activated by T-cell-derived cytokines prior to lymphocyte entry into the CNS through the blood–brain barrier (14). Indeed, severe paralysis in EAE has been observed in the absence of a significant inflammatory response (17).

The coincident expression of iNOS with the development of CNS lesions is, in theory, consistent with a number of the

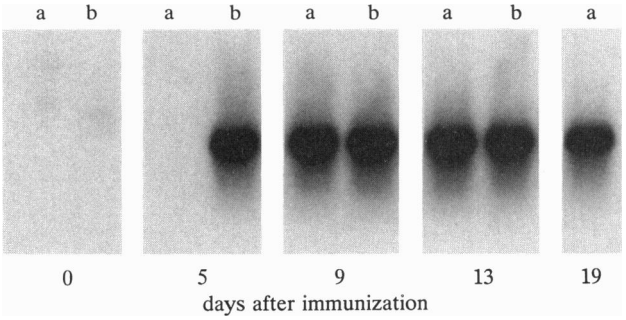

FIG. 4. Appearance of iNOS mRNA in brain tissue of rats after induction of EAE. Rats were injected with guinea pig myelin basic protein. At different days after injections, total RNA was extracted from the cerebrum and subjected to RT-PCR analysis as described in Fig. 1. The results displayed represent RT-PCR products from two rats (a and b) at each time point.

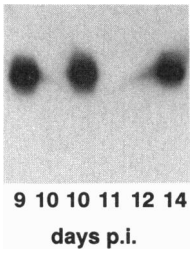

FIG. 3. Appearance of iNOS mRNA in brain tissue of mice after infection with the street strain of rabies. The total RNA was extracted from the cerebrum and subjected to RT-PCR analysis as described in Fig. 1. The results displayed represent RT-PCR products from one mouse at each time point.

cytotoxic properties ascribed to either NO or its derivative species (4). Indeed, the recent observations that (*i*) pathologic sequelae of influenza virus infection can be suppressed by superoxide dismutase (18, 19) and (*ii*) NO can react with superoxide to generate the hydroxyl radical via the generation of peroxynitrate (20) support the contention that iNOS induction in viral disease states could exert pathologic effects. However, caution must be exercised before attributing only damaging effects to the iNOS system. That is, Nathan (4) recently postulated that NO may participate in host defense mechanisms by exerting antiviral effects via the inhibition of ribonucleotide reductase. Furthermore, recent reports indicate that NO exerts tissue-salvaging effects in acute tubular necrosis (21) or hepatic damage in a murine model of endotoxin-induced shock (22). Additional studies with NOS inhibitors will be required to determine the balance between the beneficial and detrimental attributes of NO. In either case, these studies should have important implications for human disease states.

The mechanism of damage of cellular elements in several chronic neurologic diseases of man, particularly in multiple sclerosis, is virtually unknown. For example, it is highly improbable that precipitous destruction of oligodendrocytes in multiple sclerosis can be attributed to a direct infection of the cells by a hitherto unknown virus or by cytolytic T lymphocytes. Other causes have to be sought to explain the periodic and sudden onset of cell destruction leading to the formation of a plaque. The loose term "neurotoxin" has been applied in literature (23) to substances that may contribute to the mechanism of cell loss in multiple sclerosis. We feel that NO, particularly that produced by iNOS, may be a potential candidate for the toxin responsible for cell damage.

Although human iNOS has not yet been cloned, recent studies clearly indicate that this system is operative *in vitro* (24, 25) and *in vivo* (23, 26, 27). Characterization of the roles that the iNOS system plays in viral- or immune-mediated insults to human CNS could have important implications for the development of therapeutic interventions in a wide variety of neurologic disease states.

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