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Junin Virus-Induced Astrocytosis Is Impaired by iNOS Inhibition

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Because Junin virus (JV) experimental encephalitis of mice and rats is characterized by mild histopathological changes that do not seem to justify per se lethality after intracerebral infection, such a murine model seems adequate to investigate the potential role of inducible nitric oxide synthase (iNOS) as a pathogenic factor. Concomitant with a predominant astrocyte reaction, increased immunoperoxidase expression of iNOS, mitochondrial superoxide dismutase (SODm) and glutathione peroxidase (GPX) was disclosed in brain of mice infected with JV strain #44. When specific inhibition of iNOS was achieved by intraperitoneal administration of amino guanidine (AG), significantly greater mortality was observed in treated animals (70% vs. 40%), together with similar infective titers ($\sim 10^7$ PFU/g) but lower astrocytosis, as shown by glial fibrillary acidic (GFAP) labeling. As regards SODm and GPX immunochemical expression in neurons, no differences were found between mice with or without AG treatment. The present results suggest that the apparent protective role of nitric oxide (NO), when synthesized by iNOS, is unrelated to reduced viral replication but rather to enhanced astrocyte activation behaving as a beneficial cell response to virus-induced CNS damage. ***J. Med. Virol. 69:145–149, 2003.***

KEY WORDS: viral encephalitis; immunoperoxidase; GFAP, nitric oxide; superoxide dismutase; glutathione peroxidase

INTRODUCTION

The relevance of nitric oxide (NO) in the pathogenesis of viral infection has been increasingly recognized in recent years. When synthesized by the inducible enzyme nitric oxide synthase II (iNOS), NO is thought to play a major role in host response, exerting protective as well as deleterious actions [Parkinson et al., 1997]. In this connection, it has been shown that in rodent brain, iNOS induction occurs in a variety of viral infections [Schwarz, 1996; Peterhans, 1997; Akaike and Maeda, 2000]. Such iNOS expression correlates with the severity of clinical signs, the presence of macrophages in the brain, and the concomitant decrease in neuronal nitric oxide synthase expression in a time-dependent manner [Koprowski et al., 1993; Zheng et al., 1993]. These findings suggest that both excessive NO generation by activated macrophages or microglia, as well as decreased NO production in neurons may contribute to the pathogenesis of neurotropic virus infections by inducing cell oxidative stress.

Although Junin virus (JV)-induced experimental encephalitis of mice [Lascano and Berría, 1983] and rats [Lascano et al., 1989] is characterized by marked astrogliosis disclosed by glial fibrillary acidic protein (GFAP) labeling, such overwhelming astrocyte activation fails to correspond with the slight morphological changes in viral antigen-laden neurons and the mildness of inflammatory reaction. Therefore, it was of interest to resort to the mouse model to investigate whether there is increased expression of iNOS and, if so, to determine the effects of its specific inhibition by using amino guanidine (AG) [Griffiths et al., 1993]. To estimate the contribution of other enzymes related to cell oxidative balance, immunochemical labeling of mitochondrial superoxide dismutase (SODm) and cytoplasmic glutathione peroxidase (GPX) was also carried out.

Our approach disclosed a remarkable induction of iNOS, SODm, and GPX in brain of infected mice, correlating with astrocyte activation. Furthermore, AG-treated animals showed significantly increased mortality, with similar viral titers in brain homogenates when compared with untreated infected animals, although concomitant astroglial response was markedly reduced.

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MATERIALS AND METHODS

Virus

The attenuated JV strain XJ#44 [Albarino et al., 1997] was kindly provided by Dr. A.M. Ambrosio, Instituto Nacional de Enfermedades Virales Humanas Dr. J.I. Maiztegui (INEVH), Administration de Laboratorios e Institutos de Salud, Pergamino, Argentina, and then passaged twice in Vero cell cultures before stock preparation.

Animals

BALB/c mice were obtained originally from the Academia Nacional de Medicina, Buenos Aires, Argentina. Two-day-old animals were inoculated intracerebrally with 0.02 ml of virus containing approximately 10^3 plaque forming units (PFU). Control mice received equal volume of mock-infected Vero cell supernatants. All animals were given water and food ad lib.

Experimental Design

A total of 80 mice were equally split into 4 groups: A) JV-intracerebrally inoculated animals; B) as the latter plus intraperitoneally administrated AG twice daily at 130 mg/kg weight; C) intracerebrally inoculated animals with mock infected Vero cell supernatants; and D) as the latter plus AG as B. In addition to spontaneous mortality, groups of three mice were killed at 7, 15, and 30 days post-inoculation (pi) and their brains harvested. Routinely, one hemisphere was frozen at -70°C for a later infectivity assay and the other fixed with methanol plus 5% acetic acid for histologic examination and immunoperoxidase labeling.

To check the effectiveness of AG treatment, serum nitrate/nitrite levels measured by Griess reaction failed to exhibit any additional increase (data not shown).

Viral Infectivity

Brain tissues were pooled by day of collection, homogenized in a vortex in 20% PBS, clarified by centrifugation at 2,000 rpm for 15 min, and maintained at -70°C until samples were titrated by PFU on Vero cells as described previously [Gómez et al., 1991]. Briefly, after 1 hr incubation at 37°C with respective inocula, a mixture 1:1 of MEM 2 × and methylcellulose 1.6% plus 4% fetal bovine serum was added to cell monolayers. Plates were kept at 37°C for 6–7 days, and then incubated for 1 hr with a fixative-staining solution of formaldehyde and crystal violet.

Histologic Examination

Paraffin-embedded sections from fixed brain samples were stained with hematoxylin and eosin (H&E).

Immunoperoxidase Labeling

A pool of monoclonal antibodies against JV [Sánchez et al., 1989] was kindly provided by C.J. Peters, (Centers for Disease Control and Prevention, Atlanta) and used as primary antibody, as well as commercially available antibodies such as a/GFAP (Dako, Copenhagen, Denmark), a/iNOS (Cayman, Ann Arbor, MI), a/SODm and a/GPX (The binding site). Second and third antisera were biotinylated anti-species and peroxidase-conjugated streptavidin (Dako), respectively. Development reaction was achieved with 0.03% DAB (Fluka) plus 0.02% hydrogen peroxide.

Statistical Analysis

Mortality data and infectivity titers were analyzed with Fisher *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

Clinical Course

As Figure 1A shows, the XJ#44 strain of JV induced almost 40% of mortality (41/15) by Day 15 pi in Group A, whereas in animals that received additional AG treatment (Group B), it reached almost 70%. Spontaneous death was not observed in uninfected animals (Groups C, D).

Viral Infectivity

Peak infectivity titers in brain tissues were recorded at Day 7 pi (8.5×10^6 PFU/g), to gradually decrease toward the end of the observation period (Day 30 pi). Although in AG-treated mice infectivity titers were slightly higher than in mock-treated animals, no significant difference was found (Fig. 1B).

Histological Findings and Immunoperoxidase Labeling Profiles

According to HE-stained samples, signs of mild meningoencephalitis such as brain congestion, perivascular cuffing of polymorphs and mononuclear cells, and moderate cerebral infiltration of inflammatory cells were observed up to the end of the observation period (data not shown). Immunoperoxidase labeling of viral

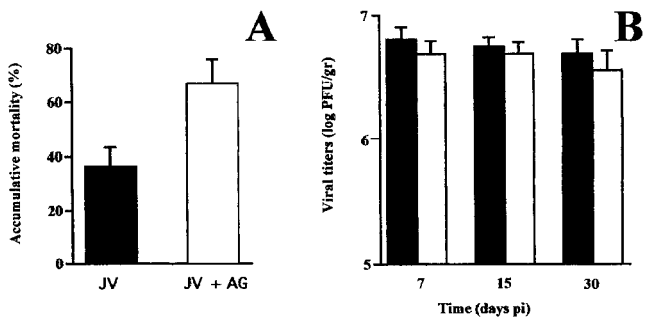

Fig. 1. Comparison between mock-treated (JV) vs. amino guanidine-treated (JV + AG) mice after infection with XJ 44-strain of Junin virus. **A:** Accumulative mortality at Day 15 pi is significantly greater in AG-treated animals ($P < 0.05$). **B:** Brain infectivity titers show a declining trend in both experimental groups (P = NS). Each time point represents a pooled tissue sample from 5 mice.

antigen demonstrated wide distribution throughout the brain of inoculated mice with or without AG treatment. Despite JV antigen deposited mainly in neurons, no necrosis or cell changes were apparent. Although to a much lesser extent, some astrocytes were also labeled (Fig. 2A). As detected by GFAP staining at Day 30 pi, widespread astrocyte hyperplasia and hypertrophy were observed (Fig. 2C), although such cell response seemed less evident in AG-treated mice (Fig. 2D). Whereas scanty iNOS labeling was observed in inflammatory cells, its expression was enhanced in astrocytes (Fig. 2B) regardless of AG treatment. In turn, SODm and GPX staining in neurons also was increased in samples from infected animals (Fig. 3B and D, respectively), when compared with uninfected animals (Fig. 3A,C), with or without AG treatment.

DISCUSSION

According to our results, the XJ#44 strain induced lower mortality than other attenuated strains derived from its more virulent ancestor XJ (prototype) such as XJ-CL3 [Winocur et al., 1990]. Therefore, the milder virulence of XJ#44 allowed many mice to survive up to Day 30 pi, enabling us to harvest their brains at an advanced stage of astrocyte activation.

In agreement with findings provided by experimental mouse infection with other JV strains [Taratuto et al., 1973; Lascano and Berría, 1974; Rabinovich et al., 1987; Medeot et al., 1990], histopathology after IC inoculation of XJ#44 strain failed to explain per se the induced high mortality. In this connection, a thymus-dependent mechanism in the development of lethal encephalitis affecting immunocompetent mice has been demonstrated by means of neonatally thymectomized [Nota et al., 1977] or congenitally athymic mice [Weissenbacher et al., 1983], supporting the involvement of cell immune response in the pathogenesis of neurological disease.

Because AG treatment increases mortality in JV-inoculated mice, such findings indicate a protective role played by NO in the course of viral infection. In another member of the *Arenaviridae* family, a correlation between neuropathology and iNOS brain expression has been described for lymphocytic choriomeningitis virus (LCMV)-infected euthymic but not athymic mice, suggesting that such difference is attributable to the presence of iNOS, only found in inflammatory infiltrate cells in the former [Campbell et al., 1994]. Relevantly, an exacerbation has been observed in AG-treated mice, though without significant differences in CNS infectivity titers in treated vs. untreated infected mice [Campbell, 1996]. More recently, these findings could only be reproduced partially when mice with a targeted disruption in the iNOS encoding gene were used [Bartholdy et al., 1999]. AG ameliorates experimental murine autoimmune encephalomyelitis [Cross et al., 1994] as well as brain infection induced by rabies virus [Ubol et al., 2001], but increases mortality of Japanese encephalitis virus-inoculated mice [Saxena et al., 2001]. In fact, NO from iNOS may not only exert contrasting effects on virus replication but also on the elicited immune response, perhaps because NO is reported to switch Type 1 helper T-cell-dependent to Type 2 helper T-cell-biased immunological response [Akaike, 2001], suggesting that the role of NO in the pathogenesis of both autoimmune and virus-induced CNS diseases is far

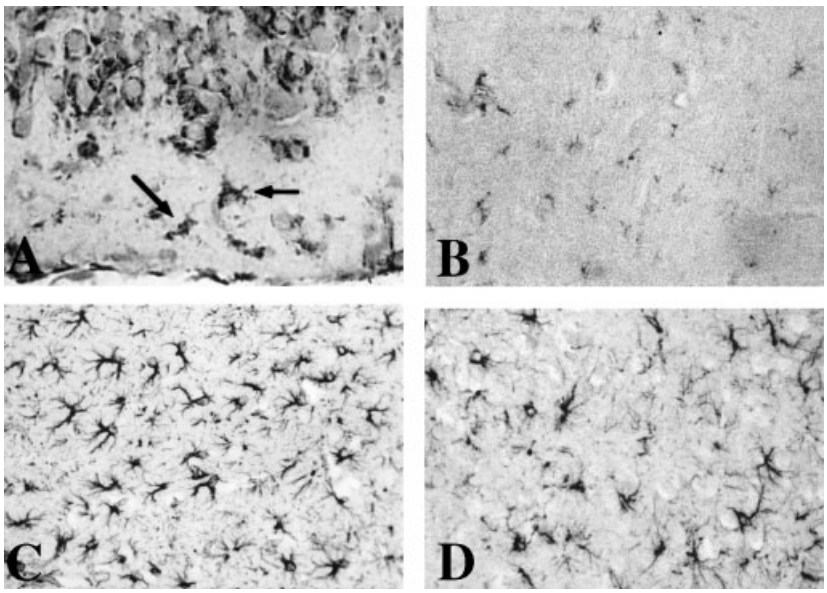

Fig. 2. Brain tissues of JV-infected mice. Immunoperoxidase labeling. **A:** Plentiful locations of viral antigen in cytoplasm of cortical neurons. Although to a lesser extent, it was also present in molecular layer astrocytes (arrows). Magnification = 450×. **B:** iNOS-immunostained astrocytes throughout brain hippocampus. Magnification = 350×. **C:** Comparison of GFAP-labeling in infected (C) and AG-treated infected mice (**D**). Magnification = 350×. As shown by increased number and cell enlargement, astrocytosis is enhanced in samples from JV-inoculated untreated animals.

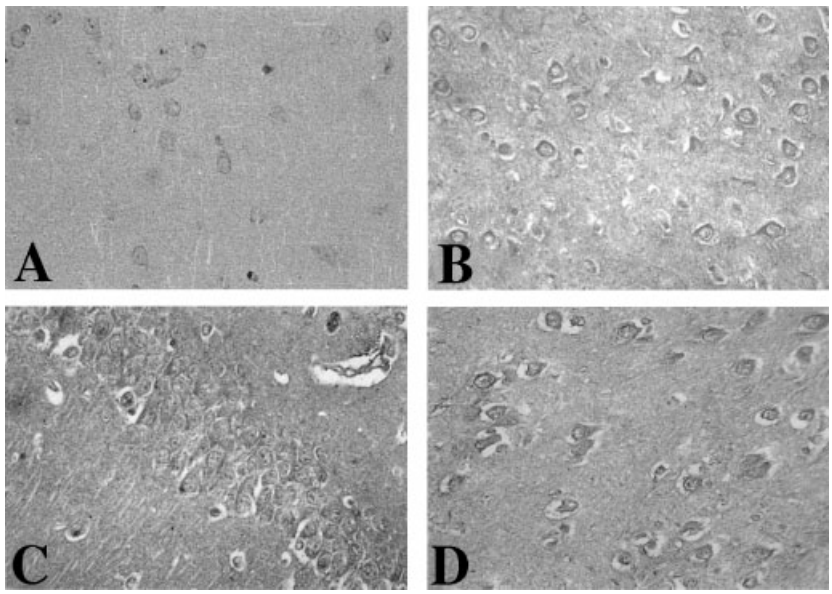

Fig. 3. In brain neurons from infected mice, immunoperoxidase labeling of both SODm (**B**) and GPX (**D**) seems enhanced as compared with their age-matched uninfected controls (**A** and **C**, respectively). Magnification = 350 ×.

from simple and still hampers therapeutic applications [Bogdan, 2001].

Our JV-infected animals showed enhanced expression of SODm and GPX, mainly in neurons. Because it is known that reactive oxygen species (ROS) play a major role in pathogenesis during viral disease [Akaike et al., 1998], it is not unlikely that such overexpression counteracts the deleterious effect exerted by generated ROS. In this regards, it has been reported that copper/zinc SOD, an isoenzyme located in cytoplasm, delays neuronal apoptosis induced by ROS [Greenlund et al., 1995], and that GPX overexpression in a model of amyloid beta-induced neurotoxicity has been shown to increase neural cell survival, thus highlighting GPX potential to reduce damage to neurons [Barkats et al., 2000]. Given the scanty data in this regard, further studies are mandatory to gain a deeper insight into the role of both enzymes in viral pathogenesis.

Recognized as one of the earliest and most remarkable responses to CNS damage, astrocytosis is evidenced by an increase in size and number of GFAP-expressing cells [Eng et al., 2000]. In our experimental model, we confirmed the presence of widespread astrocyte reaction in infected animals. In this connection, JV induction of astrocyte activation has also been described in rat brain cell cultures [Berría and Lascano, 1985] and such increased cell maturation proved concomitant with transient enhancement of both phagocytic activity [Iacono et al., 1991] and GFAP immunochemical profile [Iacono et al., 1995]. Bearing in mind that greater mortality was observed in AG-treated mice displaying lower astrocyte reactivity, as appreciated by cursory inspection, though viral infectivity titers were similar regardless of treatment, intense astrocyte activation seemed as mainly beneficial, likely by exerting supportive functions or even by restoring damaged tissues.

On the basis of our highly encouraging experimental model, the putative protective role of NO may well be related to enhanced astrocyte response, whose mutual correlation deserves a thorough study of involved molecular mechanisms.

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