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Astrocyte response to Junín virus infection

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ABSTRACT

In a previous study of experimental murine encephalitis induced by Junín virus (JV), an arenavirus, we showed increased expression of iNOS by unidentified cells, concomitant with the astrocyte reaction. The specific inhibition of iNOS was associated with greater mortality but lower astrocytosis, suggesting that the protective role of nitric oxide (NO) synthesized by iNOS was related to enhanced astrocyte activation, representing a beneficial cellular response to virus-induced central nervous system damage. In the present work, cultured astrocytes were used to study whether JV infection could trigger iNOS expression and assess its eventual relationship with viral replication, glial fibrilary acidic protein (GFAP) expression levels and the presence of apoptosis. We found that JV infection of astrocytes did not induce apoptosis but produced both increased iNOS synthesis, detected by immunocytochemistry and fluorescence activated cell sorting (FACS) analysis, and increased NO, which was indirectly measured by nitrite/nitrate levels. These changes occurred early relative to the increases in GFAP expression, as detected by immunocytochemistry, FACS analysis and RT-PCR. The fact that iNOS inhibition abolished enhanced GFAP expression in infected monolayers suggests that NO was directly involved. In addition, iNOS inhibition enhanced virus replication. Together with data from confocal microscopy, these results suggest that JV induces iNOS expression in infected astrocytes and that the resulting NO has an important role both in reducing viral replication and in enhancing subsequent astrocyte activation.

Astrocytes are the most numerous cell type in the central nervous system (CNS). They provide structural, trophic and metabolic support to neurons, in addition to modulating synaptic plasticity and activity, neurite outgrowth and neuron regeneration [8].

An important characteristic of astrocytes is their activation, characterized by enhanced expression of glial fibrilary acidic protein (GFAP), in response to diverse CNS injuries such as trauma, infectious diseases or chemical insults. The prominence of this reaction and its evolutionary conservation indicate that activated astrocytes serve important functions in the injured CNS [9] by selective expression of several proteins including inducible nitric oxide synthase (iNOS) [14]. Remarkably, both beneficial and detrimental effects have been attributed to reactive astrocytes [8,18,19].

Argentine hemorrhagic fever (AHF) is a systemic febrile syndrome characterized by several haematological alterations caused by Junín virus (JV), a member of the *Arenaviridae* [26]. Patients with AHF frequently exhibit neurological involvement in the acute period [10,20]. In addition, although serum treatment reduces mortality from 30% to 1%, about 10–15% of patients who receive this treatment present a delayed neurological syndrome [28]. Interestingly, the histopathological findings in both humans as well as animal models do not reflect the severity of disease [20], and although it has been shown in animals that the virus can reach the CNS via a neural route [17], its pathogenesis is poorly understood. When studied in experimental models, viral antigen can be detected in the CNS during the acute period, widely disseminated primarily in neurons and a few astrocytes [16]. The chronic stage is characterized by the gradual disappearance of viral antigen and a prominent astrocyte reaction [16]. In a previous study of JV-induced experimental murine encephalitis, we showed increased expression of iNOS by unidentified cells, concomitant with the astrocyte reaction. Specific inhibition of iNOS was associated with greater mortality but lower astrocytosis. Still, similar infective titers were observed, suggesting that the apparent protective role of nitric oxide (NO) synthesized by iNOS was unrelated to reduced viral replication but was rather due to enhanced astrocyte activation, representing a beneficial cellular response to viral-induced

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CNS damage [12]. Our present work is aimed to show if JV infection could trigger expression of iNOS in astrocytes and whether subsequently iNOS expression affects viral replication, astrocyte apoptosis or astrocyte activation. This study used an *in vitro* model, which affords the advantage of avoiding the complexity of CNS microenvironment and the host immune response. To this end, we used JV-infected cultured astrocytes to study the relationship between viral replication and the expression levels of iNOS and GFAP.

The JV strain P3441 and the infectivity titration procedure have been previously described [11]. UV-irradiated JV was used as a negative control.

Astroglial cell cultures were obtained from the brains of newborn Balb/c mice as previously described [2]. Briefly, cortical hemispheres were harvested, and neural tissues digested by 0.25% trypsin. Flasks were seeded with 6×10^5 to 8×10^5 cells/ml of growth medium (D-MEM plus 10% fetal calf serum) twice for 1 h in order to eliminate fibroblast contamination. By changing the supernatant within 24 h, the neurons were eliminated by means of differential attachment, leaving an almost homogenous population of glial cells. After 3 weeks at 37 °C, the already confluent primary cell culture was shaken for 2 h at 37 °C, and supernatant was discarded in order to detach contaminating oligodendrocytes growing on top of the remaining adherent astroglial cell monolayer, which in turn were trypsinized. The resulting cells were then resuspended (3×10^5 cells/ml of growth medium) and seeded as first subculture in 24 wells plates with 12 mm glass inserts or in 25 cm^2 flasks. Subsequently, immunoperoxidase labeling for GFAP showed a highly homogeneous population of astrocytes ($\geq$ 95%, data not shown).

After 2 days of seeding, growth medium was removed, and cell monolayers were infected with JV at a multiplicity of infection of 1. At 1, 3, 7 and 14 days post-infection (PI), 3–4 samples were harvested for each experimental time point. Supernatants were routinely frozen at −70 °C for later measurement of infectivity titers and nitrate/nitrite levels. Monolayers were fixed with 2% paraformaldehyde plus 0.1% Triton X-100 at 37 °C for immunolabeling or trypsinized for cytofluorometry. In some studies, astrocytes were treated with the iNOS specific inhibitor, *N*6-(1-Iminoethyl)-L-lysine hydrochloride (L-NIL) (5 μM) from Tocris (USA).

The DNA content of astrocytes was analyzed by flow cytometry using propidium iodide and histogram analysis to identify hypodiploid nuclei as previously described [23]. Morphological changes and viability were evaluated by staining cells with a mixture of fluorescent DNA-binding dyes: acridine orange (100 μg/ml) to determine the percentage of cells undergoing apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. At least 200 cells were scored in each experiment. Samples treated with H_2O_2 (400 mM) for 1 h served as a positive control.

A pool of monoclonal antibodies against JV [22] was kindly provided by Dr. C.J. Peters (Centers for Disease Control and Prevention, Atlanta, USA) and used as a primary antibody, as well as commercially available antibodies such as anti-GFAP (Dako Corporation, CA, USA) and anti-iNOS (Cayman Chem. Co., MI, USA). For immunoperoxidase labeling, the second and third reagents were biotinylated anti-species immunoglobulin and peroxidase-conjugated streptavidin (Dako), respectively. Development of the reaction was done with 0.03% DAB (Fluka, Buchs SG, Switzerland) plus 0.02% hydrogen peroxide. For fluorescence microscopy, the secondary immunoglobulins were FITC-conjugated anti-rabbit (Dako, USA) and Cy3-conjugated anti-mouse Ig (Zymed, USA). No primary antibody was used as a negative control of the procedure. Analysis was performed using a confocal laser-scanning microscope (LSM 510; Carl Zeiss MicroImaging, Inc.) or an inverted microscope (Eclipse TE2000-U; Nikon) equipped with an oil immersion plan Apo 60× NA 1.4, 100× NA 1.4 or a dry plan Fluor 40× NA 0.6 objective (Nikon) and a CCD camera (MicroMAX RTE/CCD-1300Y; Princeton Instruments). Acquisition and analysis of still images were performed using MetaMorph Imaging Software (version 6.1; Universal Imaging Corp.).

After trypsinization, astrocytes were centrifuged at 200 × *g* for 10 min, resuspended in PBS/FBS and incubated with the appropriate primary antibody and a FITC-conjugated fragment $F(ab')_2$ anti-species IgG (Immunotech, Marseille, France). For non-specific binding, FITC-conjugated or primary MoAbs were replaced by an irrelevant isotypic MoAb. After labeling, cells were washed, fixed with 1% paraformaldehyde and analyzed by fluorescence activated cell sorting (FACS) analysis.

Nitrite sample concentration was established by the Griess reaction as described [12]. Optical density at 550 nm was determined on a spectrophotometer from a sodium nitrite (Sigma) curve performed for each experiment. Medium absorbance alone was subtracted from the value obtained for each sample.

Total RNA was isolated from cell pellets using *Trizol* (Invitrogen) as recommended by the manufacturer. cDNA was synthesized from 20 ng of total RNA using 15 μM random hexamers and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's protocol. The cDNA samples were diluted 10-fold, and the PCR reaction was conducted with an annealing temperature of 55 °C using the following primers (F/R): βActin ACTATTGGCAACGAGCGGTT/CAGGATTCCATACCCAAGAAGGA; GFAP AAAACCGCATCACCATTCC/CCTCTTCCCTTCCAATTCTAAC. All reactions were confirmed to be in the linear range of amplification.

All results are expressed as means ± S.E.M. Data analysis was accomplished using a Student's *t*-test. Values of p lower than 0.05 were considered statistically significant.

Infectivity titration of the culture medium supernatants from infected cells ranged from 10^5 to 10^6 plaque forming units (PFU)/ml at 3, 7 and 14 days post-inoculation (PI) (the end of the observation period), peaking at day 7 (3×10^6 PFU/ml, $n = 3$) (Fig. 1A). No plaques were observed after infectivity titration of supernatants from UV-irradiated JV infected cells (data not shown). Infected astrocytes observed by phase contrast microscopy showed no cytopathic effects (data not shown). Eventual apoptosis was assessed later by morphological and flow cytometry analysis. No differences were found in control (5.8 ± 0.7% of apoptotic cells, $n = 4$) vs. infected cells (5.2 ± 0.8% of apoptotic cells, $n = 3$) (Fig. 1B) in contrast to H_2O_2-treated astrocytes (78 ± 7% of apoptotic cells, $n = 4$) (Fig. 1B). JV infection was evident in the cell monolayer from day 3 PI by immunostaining of viral antigens (Fig. 1C) and was clearly more intense when iNOS was inhibited by L-NIL (Fig. 1D). This result was confirmed by flow cytometry analysis (Fig. 1E) and viral infectivity titration of cell culture supernatants (Fig. 1A).

Enhanced iNOS expression, detected by immunoperoxidase staining and flow cytometry analysis, was first evident from day 1 PI and was more intense at day 3 PI in infected cultured astrocytes compared with UV-irradiated JV-infected controls (Fig. 2A and C, $n = 3$). Confocal microscopy analysis showed localization of viral and iNOS antigens at the same infected cells, in most cases (Fig. 2B). In addition, supernatants from JV-infected astrocytes showed a marked increase in NO production from day 3 to 7 PI (38 ± 2 nmol/mg prot vs. 18 ± 1 nmol/mg prot at day 3, $p \leq 0.05$, $n = 4$; 82 ± 12 nmol/mg prot vs. 19 ± 1 nmol/mg prot at day 7, $p \leq 0.05$, $n = 4$, JV-infected and UV-irradiated), which was abolished when an iNOS inhibitor was added (20 ± 1 nmol/mg prot at day 3 and 22 ± 2 nmol/mg prot at day 7) (Fig. 2D, $n = 4$).

In order to investigate whether JV infection *per se* caused astrocyte activation, we performed confocal microscopy analysis to see if the expected enhanced staining of GFAP co-localized with viral antigens over time. Results showed no difference at day 1 PI and a

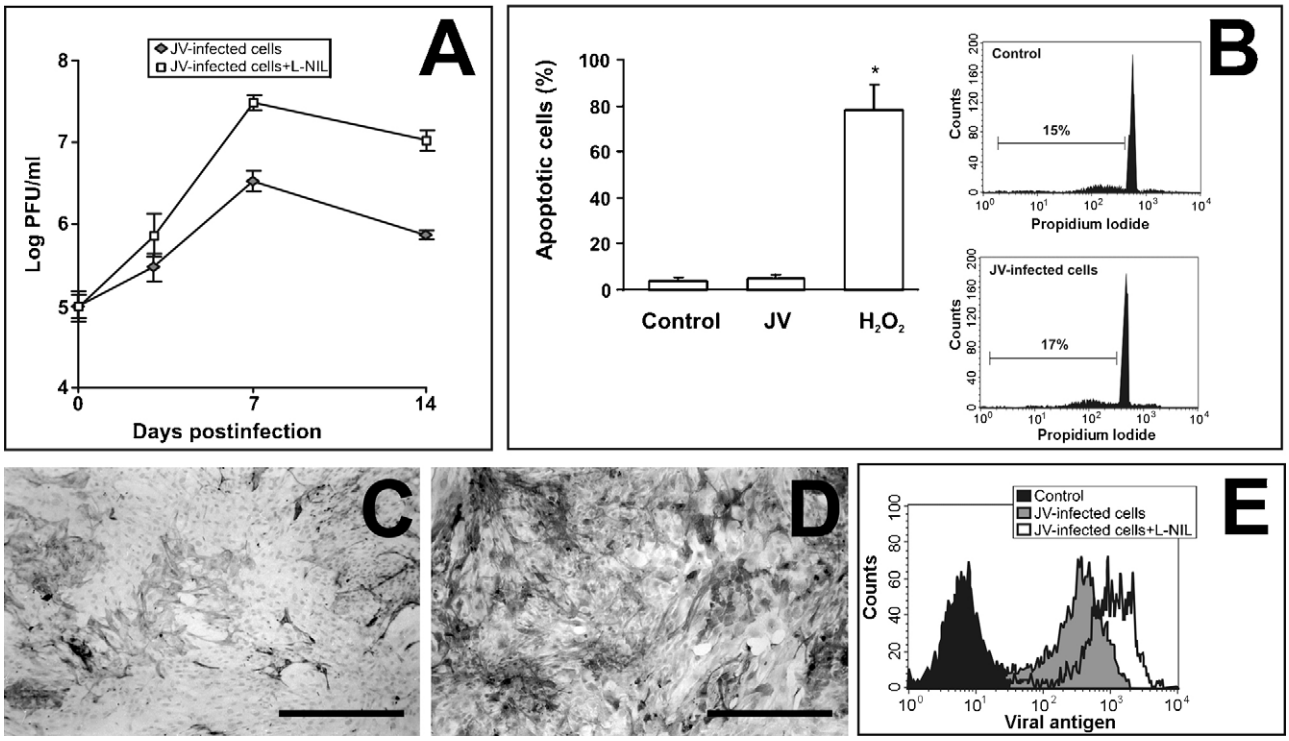

Fig. 1. JV infects astrocytes in the absence of apoptosis. Infectivity titers of supernatants were determined as previously described [11] and expressed as PFUs. The titers were higher in L-NIL-treated monolayers compared with those from the untreated cells ($n = 3$) (A). Morphology and cytometry analysis showed no differences in apoptosis between JV-infected cells and UV-irradiated JV-infected cells (control) ($n = 3$) (B). Immunoperoxidase labeling of JV-infected cultures showed cells exhibiting presence of viral antigen (C), which were enhanced in L-NIL-treated monolayers (D), as confirmed by FACS analysis (E). Scale bar C and D: 400 μm.

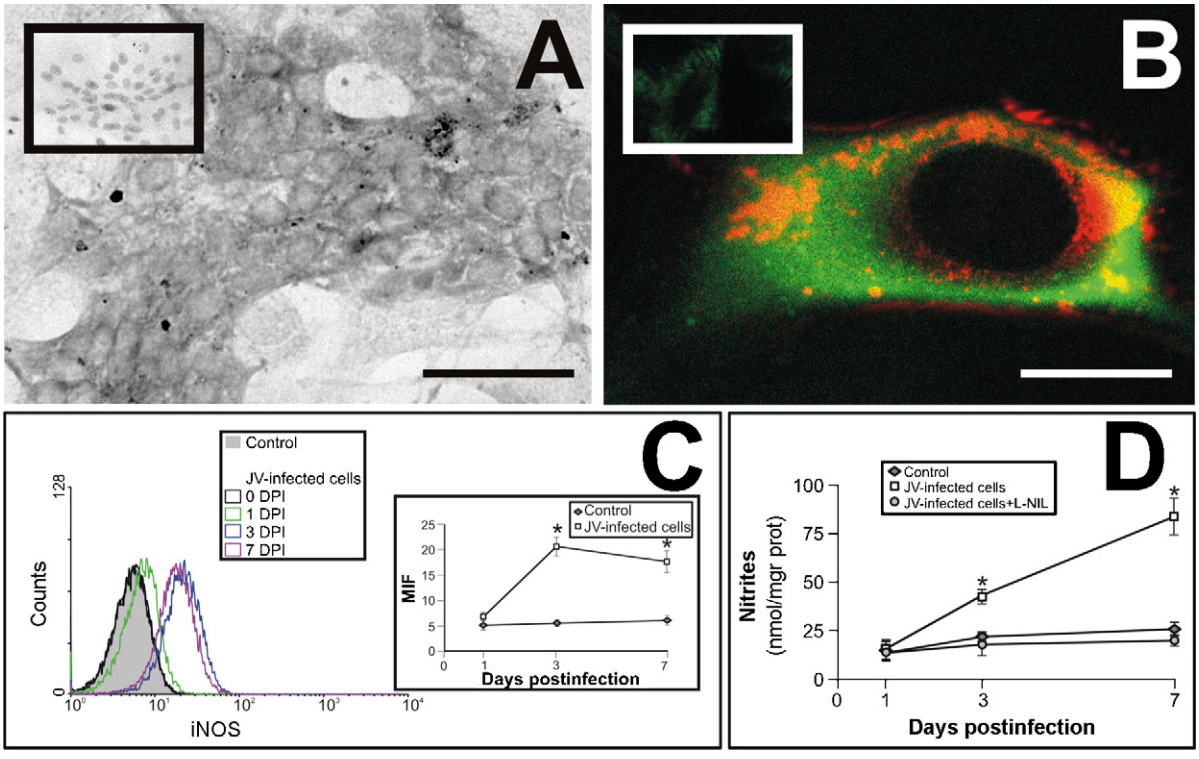

Fig. 2. JV infection enhances NO production by induction of iNOS expression. Immunoperoxidase labeling showed enhanced iNOS detection in JV-infected astrocytes (A) compared with UV-irradiated JV-infected cells (A inset). A representative confocal microscopy image revealed JV (red) and iNOS (green) antigens present in the same JV-infected astrocyte (B), in contrast of UV-irradiated JV-infected astrocytes that only showed a slight iNOS presence (B inset). FACS analysis of iNOS expression in JV-infected or control cells over time were shown as overlapping histograms of one representative experiment on the left with the mean fluorescence intensity (MFI) in arbitrary units on the right ($n = 4$) (C). As evaluated by Griess reaction, increased nitrite concentration in supernatants from infected cultures as compared to controls, was abolished after L-NIL treatment (D). Scale bars A: 50 μm and B: 8 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

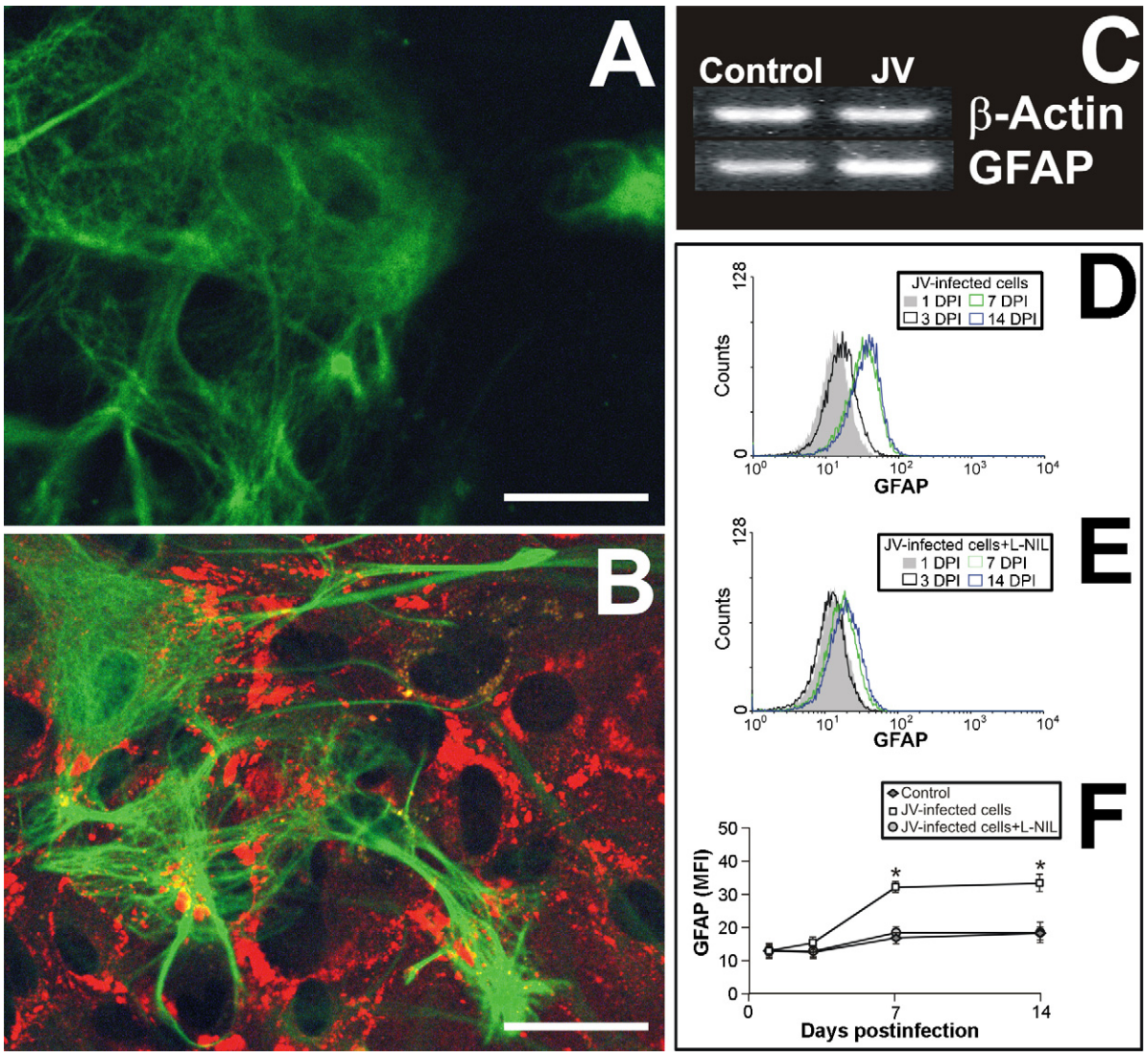

Fig. 3. JV infection induces activation of astrocytes. Confocal microscopy of UV-irradiated JV-infected astrocytes showed GFAP staining (green) in the absence of viral antigens. In contrast, JV-infected astrocytes showed an enhanced GFAP expression with lack of co-localization of GFAP and viral antigens (red) (A and B, respectively). A representative RT-PCR of four experiments showed that JV infection correlated with an increase in GFAP mRNA transcription (C). FACS analysis of GFAP expression in JV-infected and L-NIL treated cells over time were shown as overlapped histograms of one representative experiment (D and E) with the mean fluorescence intensity (MFI) in arbitrary units at the right ($n = 3$, with UV-irradiated JV-infected astrocytes as control) (F). Scale bar A and B: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

slight difference at day 3 PI when compared with matched controls represented by UV-irradiated infected astrocytes (data not shown). In contrast, GFAP expression was observed in the monolayers as foci that were clearly increased in number and stain intensity in infected cultures at day 7 and 14 PI with lack of co-localization between viral antigens and GFAP (Fig. 3A and B). The enhanced GFAP staining correlated with augmented transcription of its mRNA at the same time points (Fig. 3C) and was completely abolished when cells were pretreated with the iNOS inhibitor L-NIL. These results were confirmed by flow cytometry (Fig. 3D–F, $n = 3$).

Although JV replication in brain astrocytes was originally demonstrated more than three decades ago [15], the role played by these cells in the pathogenesis of experimental JV-induced encephalitis has remained unclear, including the mechanism of astrocyte activation by JV. Since there were no alterations in cell morphology or apoptosis – keeping in mind that both infected and non-infected subconfluent cell monolayers reached confluence at the same time (data not shown) – it may be inferred that the astrocyte cell cycle was unaffected by JV replication, contrary to findings in other cell types such as Vero cells [7]. In contrast, JV infection increased iNOS synthesis and the resulting level of NO. The temporal correlation between the detection of viral and iNOS antigens, co-localization of both antigens and the lack of enhanced expression of iNOS in UV-irradiated JV monolayers suggest that iNOS expression is one of the early astrocyte responses to JV replication. In contrast, the marked increase in GFAP was delayed compared with iNOS expression, and there was no co-localization of viral and GFAP antigens on days 3–7 PI. In addition, the concomitant enhanced expression of GFAP mRNA rules out that this result is due to the previously described transient soluble detection fraction of GFAP following JV infection [13]. Moreover, GFAP expression was observed in the monolayer as foci that were increased both in number and intensity over time, suggesting a paracrine mechanism as the cause of such astrocyte activation. The fact that iNOS inhibition abolished the enhanced GFAP expression in infected monolayers suggests that NO is directly involved. This is in agreement with the

demonstrated role of NO in the expression of GFAP by astrocytes via GC-cGMP-PKG [4] and with the increased NO production and GFAP expression observed in influenza virus-infected mice [27]. Using cytokine production as a parameter of astrocyte activation, it was recently shown that glial cells, including astrocytes, were activated by *Lymphocytic Choriomeningitis Virus* (LCMV) in a TLR2-MyD88/Mal-dependent manner [29]. Although LCMV and JV have a different pathogenesis, both are arenaviruses and share the same genomic organization [21]; thus, it seems probable that JV activate astrocytes in a similar manner. In contrast, a marked increase in the expression of GFAP in the absence of iNOS expression has been found to be related to the neurovirulent properties of Sindbis virus strains [5]. Thus, intense viral-induced astrocyte activation can hardly be a stereotyped response to viral infection, as it may be selective with regard to concomitantly expressed molecules.

In the present study, we found that iNOS inhibition resulted in higher viral titers as well as viral antigen detection, in contrast to similar infectivity titers previously found in brain homogenates from mice treated or untreated with iNOS inhibitors [12]. The reasons for this discrepancy may be that most viral replication in the brain occurs in neurons instead of astrocytes, and it is probable that neurons do not produce NO at the same level as astrocytes. Keeping in mind these *in vivo* and *in vitro* results, it is possible to speculate that astrocytes respond early to JV infection with enhanced expression of iNOS and subsequent NO production, resulting in both reduced viral replication as well as higher astrocytosis, playing a protective role in the course of viral disease. Nevertheless, following infection of euthymic – but not athymic – mice by LCMV, a correlation between neuropathology and iNOS brain expression was described in euthymic mice, attributable to the presence of iNOS in infiltrating inflammatory cells [6]. Moreover, in Venezuelan equine virus-induced encephalitis, the inflammatory response is partially mediated by iNOS and TNF-α and seems to contribute to neurodegeneration [24]. In fact, NO production by iNOS might exert contrasting effects not only on virus infection but also on the elicited immune response [3]. This is perhaps because NO is reported to switch type 1 helper T (Th_1) cell-dependent responses to Th_2-biased immunological responses [1].

Further studies focusing on the role of astrocyte activation by NO should be carried out, as an improved understanding of astrocytes in the pathogenesis of viral-induced neurologic disease offers the potential to develop novel strategies to treat neurological disorders [25]. In this field, JV has confirmed its potential as an *in vivo* and *in vitro* experimental model of astrocyte response to viral injury.

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