Glial activation involvement in neuronal death by Japanese encephalitis virus infection

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Japanese encephalitis is characterized by profound neuronal destruction/dysfunction and concomitant microgliosis/astrogliosis. Although substantial activation of glia is observed in Japanese encephalitis virus (JEV)-induced Japanese encephalitis, the inflammatory responses and consequences of astrocytes and microglial activation after JEV infection are not fully understood. In this study, infection of cultured neurons/glia with JEV caused neuronal death and glial activation, as evidenced by morphological transformation, increased cell proliferation and elevated tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and RANTES (regulated upon activation, normal T-cell expressed and secreted) production. Replication-competent JEV caused all glial responses and neurotoxicity. However, replication-incompetent JEV lost these abilities, except for the ability to change microglial morphology. The bystander damage caused by activated glia also contributed to JEV-associated neurotoxicity. Microglia underwent morphological changes, increased cell proliferation and elevated TNF-α, IL-1β, IL-6 and RANTES expression in response to JEV infection. In contrast, IL-6 and RANTES expression, but no apparent morphological changes, proliferation or TNF-α/IL-1β expression, was demonstrated in JEV-infected astrocytes. Supernatants of JEV-infected microglia, but not JEV-infected astrocytes, induced glial activation and triggered neuronal death. Antibody neutralization studies revealed that TNF-α and IL-1β, but not RANTES or IL-6, released by activated microglia appeared to play roles in JEV-associated neurotoxicity. In conclusion, following JEV infection, neuronal death was accompanied by concomitant microgliosis and astrogliosis, and neurotoxic mediators released by JEV-activated microglia, rather than by JEV-activated astrocytes, had the ability to amplify the microglial response and cause neuronal death.

INTRODUCTION

The central nervous system (CNS) consists of an intrinsic network of neurons and glia. The CNS response to ischaemia, degenerative diseases and viral infection is largely manifested by neuronal loss and inflammatory responses (Gao et al., 2002; Liao et al., 2002; Chen et al., 2004; Raung et al., 2005; German et al., 2006; Ovanesov et al., 2006; Ghoshal et al., 2007; van Marle et al., 2007; Saxena et al., 2008; Swarup et al., 2008). Generally, the onset and/or interactive crosstalk of neuronal injury and neuroinflammation play a critical role in the pathogenesis of neurological disorders. The hallmark of neuroinflammation is the activation of resident glial cells and recruitment of peripheral immune cells, as well as the production of pro-inflammatory mediators. Activated glia are characterized by increased cell proliferation, macrophage-specific activation marker expression and bioactive mediator production, as well as by morphological changes. In the CNS, the activation of glia, including microglia and astrocytes, as a ubiquitous hallmark of different neuropathological states, is important in forming an environment that contributes either to successful repair of damaged brain tissue or to severe injury of bystander cells. Although several activated glia-derived
factors are thought to contribute to tissue repair, the majority are believed to induce neurodegeneration via mechanisms that are not yet fully understood (Giulian, 1993; Chao et al., 1995; Kong et al., 1996; Ridet et al., 1997).

Japanese encephalitis virus (JEV) belongs to the encephalitic flaviviruses and frequently causes acute encephalopathy (Chambers et al., 1990). Although JEV causes productive infection in most cell lines derived from different tissue origins, clinical and animal studies suggest that the principal target cells for JEV in the CNS are neurons (German et al., 2006; Ghoshal et al., 2007). As a result of infection, massive dysfunction and/or destruction of neurons is a hallmark of JEV-associated encephalitis (Kumar et al., 1990). Pathological features of JEV-associated encephalitis also include inflammation-related neuropathological complications. The recruitment and activation of inflammatory cells in the lesion sites and elevated levels of pro-inflammatory cytokines in the serum and cerebrospinal fluid (CSF) have been observed in Japanese encephalitis (Chaturvedi et al., 1979; Mathur et al., 1988; Khanna et al., 1991; Ravi et al., 1997; Singh et al., 2000). It has been demonstrated that the mortality rate increases with increasing concentrations of cytokines in the serum and CSF in Japanese encephalitis patients (Ravi et al., 1997). Regarding JEV-associated neuroinflammation, a number of studies have shown that JEV infects microglia and astrocytes, and their consequent activation contributes to neuronal death (Chen et al., 2000, 2004; Raung et al., 2005, 2007; Abraham & Manjunath, 2006; Bhowmick et al., 2007; Ghoshal et al., 2007; Mishra et al., 2007; Das et al., 2008; Swarup et al., 2008). These studies suggest that, in addition to direct viral infection of neurons, bystander damage caused by activated microglia/astrocytes is involved in JEV-induced neuronal death. However, the extent to which glial cells are infected and contribute to JEV-related pathogenesis has yet to be determined.

Although activation of microglia and astrocytes is found after JEV infection, activated microglia rather than activated astrocytes are proposed to mediate severe neurotoxicity (Raung et al., 2005; Ghoshal et al., 2007; Mishra et al., 2007). Currently, the discrepancy between JEV-activated microglia and astrocytes is not fully understood. Therefore, in the present study, we investigated cellular responses and alterations in microglia and astrocytes after JEV infection in order to elucidate their potential neurotoxic actions after activation.

**RESULTS**

**JEV infection causes neuronal death**

Neurotoxicity was assessed by morphological analysis and cell counting after immunocytochemical staining for microtubule-associated protein 2 (MAP-2). As shown in Fig. 1(a), JEV infection caused degeneration of neurite processes and decreased numbers of visible neurons in cultures of neurons/glia (213 ± 45 in mock vs 49 ± 21 in JEV, P<0.01). Demise of the neuronal body was not detected in UV-inactivated (225 ± 54) or boiling-inactivated (201 ± 49) JEV-infected cultures. In contrast to the apparent neuronal damage, astrocytes were not significantly affected by JEV infection. Microglia possessed a process-bearing morphology (94.2 ± 3.7 %) in neuron/glial cultures and changed into a rounded, darkly stained cell morphology (93.6 ± 4.5 %) after JEV infection (Fig. 1b). Evidence suggests that this type of morphological

![Fig. 1](http://vir.sgmjournals.org)
switch represents a transformation from resting microglia to reactive/amoeboid microglia (Ghoshal et al., 2007). These results implied that neuronal death after JEV infection was accompanied by the activation of glia, especially microglia.

**Activated glia contribute to JEV-induced neuronal death**

One of the neurotoxic mechanisms activated by glia involves triggering bystander damage. To elicit this effect, the supernatants were collected from mock- and JEV-infected mixed glial cultures 48 h after infection. JEV infection caused neuronal death in neuron cultures, as shown by the change in the number of viable neurons (194 ± 55 in control vs 43 ± 26 in JEV infection, \( P < 0.01 \); Fig. 2a). Cell damage in neuron cultures was observed after exposure to JEV-conditioned medium (31 ± 19, \( P < 0.01 \)). Intriguingly, the neurotoxic action of JEV-conditioned medium was abolished by boiling (211 ± 57), but not by UV (36 ± 21) pre-treatment, before exposure (Fig. 2a). JEV-conditioned medium showed a similar neurotoxic effect and action characteristics in neuron/glial cultures. The numbers of neurons in mock, JEV, JEV/UV and JEV/boiled cultures were 209 ± 35, 35 ± 16, 68 ± 34 and 203 ± 55, respectively (Fig. 2b). As with direct JEV infection, a morphological change in the microglia (4.9 ± 1.9% with a process-bearing morphology, 94.8 ± 3.7% with a rounded morphology) was observed in neuron/glial cultures exposed to JEV-conditioned medium when compared with mock-conditioned medium (90.3 ± 5.1% with a process-bearing morphology, 7.2 ± 3.2% with a rounded morphology) (Fig. 2b). JEV-conditioned medium pre-treated with UV still exerted a morphological effect (5.1 ± 2.9% with a process-bearing morphology, 93.8 ± 5.7% with a rounded morphology). However, boiling pre-treatment disrupted the modulatory effect of JEV-conditioned medium on microglial morphology (91.9 ± 5.9% with a process-bearing morphology, 6.8 ± 3.1% with a rounded morphology) (Fig. 2b). Thus, JEV-conditioned medium from mixed glia contained soluble mediators capable of triggering neuronal death and microglial morphological changes.

![Fig. 2. Effect of JEV-conditioned media on neural cells. The supernatants of mock-infected (Mock CM) and JEV-infected (JEV CM) mixed glial cultures were collected 48 h after infection and mixed with an equal volume of fresh medium. The conditioned media were added to cultured neurons (a) and neuron/glial cultures (b) for 24 h. JEV CM was also pre-treated with UV (JEV CM/UV) or boiled (JEV CM/boiled) before adding to the cultured cells. Cultured neurons (a) were also directly infected with JEV for 24 h (JEV). Neural cells were detected by the immunoreactivity of MAP-2 (a, b) and CD68 (b). Representative micrographs are shown. Bars, 60 μm.](image-url)
JEV infection induces morphological changes in microglia

Apparent morphological changes in microglia were demonstrated in neurons/glia that were infected with JEV or exposed to JEV-conditioned medium. To characterize the morphological effects in more detail, mixed glial, astrocyte and microglial cultures were investigated further. In mixed glial cultures, 94.9 ± 3.9 % of the microglia had a process-bearing morphology and 3.8 ± 2.1 % had a rounded morphology. JEV infection transformed the microglia from process-bearing to a rounded, darkly stained morphology (5.1 ± 2.8 % with a process-bearing morphology, 92.8 ± 3.1 % with a rounded morphology) in mixed glial cultures, similar to those composed of neurons/glia. However, it was difficult to differentiate morphological changes in astrocytes between mock- and JEV-infected cells (Fig. 3). Cultured astrocytes exhibited a polygonal, flattened morphology, and no detectable morphological changes were observed after JEV infection (Fig. 3). Intriguingly, unlike in neuron/gial cultures, microglia alone had a small, rounded cell body (~25 μm in diameter) without a process-bearing morphology under in vitro culture conditions (Fig. 3). JEV infection caused cytoplasmic expansion (more than twice the cell volume) and branches (3.1 ± 1.6 % in mock-infected vs 76.8 ± 15.4 % in JEV-infected cells; Fig. 3). To determine whether replication-competent virus was essential for the induction of these morphological changes, microglial cultures were stimulated with UV- and boiling-inactivated JEV. Boiling-inactivated JEV was unable to induce the morphological changes (2.9 ± 1.3 %), whereas UV-inactivated JEV retained the same ability to transform microglial morphology (71.4 ± 9.8) as wild-type JEV (Fig. 4). These findings implied that virus amplification is not an essential prerequisite for morphological modulation in JEV-infected microglia.

JEV infection differentially induces cytokine production in glia

The activation of glial cells yields multiple bioactive factors leading to secondary glial activation and neuronal injury (Giulian, 1993; Kong et al., 1996). To define the mechanisms involved in the initiation of a pro-inflammatory milieu in the CNS leading to eventual encephalitis, the ability of JEV to induce a pro-inflammatory mediator production was measured. The results indicated a significant upregulation of pro-inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and RANTES (regulated upon activation, normal T-cell expressed and secreted) in neuron/gial cultures after JEV infection. Inactivation of JEV by UV and boiling dramatically reduced the levels of these upregulated pro-inflammatory mediators (Fig. 6a). The results suggested that virus replication and/or productive infection is probably required for upregulation of these pro-inflammatory mediators. Intriguingly, glia showed the distinct expression profile of pro-inflammatory mediators in response to JEV infection (Fig. 6b). The expression of TNF-α, IL-1β, IL-6 and RANTES was elevated in JEV-infected mixed glial and microglial cultures. Among the four mediators, only the inductive production of IL-6 and RANTES was demonstrated in JEV-infected astrocyte cultures. These results indicated that JEV infection selectively induces expression of certain pro-inflammatory mediators in glial cells and that microglia show a relatively higher immune competence.

JEV infection induces DNA synthesis in microglia

To verify the active proliferation of cells in response to JEV infection, incorporation of 5-bromo-2′-deoxyuridine (BrdU) was determined. As illustrated in Fig. 5, elevated BrdU incorporation was detected in neuron/glial cultures exposed to JEV, whereas UV- and boiled-inactivated JEV lost this inductive activity. The ability of specific cells to respond directly to virus challenge was tested in distinct cultures after inoculation with JEV. Mixed glial and microglial cultures responded to JEV infection by elevating BrdU incorporation. In contrast, negligible proliferation was seen in astrocyte cultures after JEV infection. Thus, microglia appeared to be the dominant cells involved in active proliferation after JEV infection.

Fig. 3. Appearance of glial cells after JEV infection. Mixed glial, astrocyte and microglial cultures were mock-infected or infected with JEV for 48 h. Glial cells were detected by the immunoreactivity of GFAP and CD68. Representative micrographs are shown. Bars, 60 μm.
Microglia rather than astrocytes contribute to JEV-induced neuronal death

To elucidate the potential contribution of activated astrocytes and microglia in JEV-induced neuronal death, conditioned medium was collected from JEV-infected microglia and astrocytes. As shown in Fig. 7, conditioned medium from JEV-infected microglia (55 ± 34) rather than astrocytes (198 ± 64) provoked significant neurotoxicity in neuron cultures (mock, 215 ± 59). The above-mentioned studies demonstrated that microglia are the prominent effector cells in response to JEV infection and that their consequent activation provokes neuronal injury. What are the potential neurotoxic mediators released by JEV-infected microglia? Pro-inflammatory cytokines might be a possibility. Therefore, to assess the involvement of glia-derived pro-inflammatory cytokines in JEV-induced neurotoxicity, neutralizing antibodies against TNF-α, IL-1β, RANTES and IL-6 were used. The neurotoxic action of supernatants from JEV-infected microglia was attenuated by treatment with TNF-α or IL-1β neutralizing antibody. However, attenuation of neurotoxicity was not observed following treatment with control IgG or RANTES or IL-6 neutralizing antibody (Fig. 8). These results suggested that microglia-derived TNF-α and IL-1β are involved in neurotoxic cascades after JEV infection.

DISCUSSION

JEV-associated neurotoxicity has been well demonstrated in clinical and animal studies (German et al., 2006; Ghoshal et al., 2007). Although histochemical studies have revealed microgliosis and astrogliosis in areas of neuronal damage,
the inductive activation of microglia and astrocytes and the consequences of this activation in Japanese encephalitis have not yet been fully determined. In an animal model of Japanese encephalitis, the elevated pro-inflammatory mediator release by activated microglia was positively correlated with neuronal death, and inhibition of microglia activation resolved neuron inflammation and lethal encephalitis (Ghoshal et al., 2007; Mishra & Basu, 2008), implying a neurotoxic role of activated microglia. The causative effect of activated microglia in JEV-associated neuronal damage was demonstrated further by in vitro cell line studies. JEV infection-stimulated and cytokine-activated microglial cell lines produced neurotoxic mediators that caused neuronal death in neuronal cell lines (Ghoshal et al., 2007; Das et al., 2008; Swarup et al., 2008). In parallel with these findings, the present study elicited the inductive characteristics of glia by JEV infection and showed the neurotoxic potential of activated microglia via primary culture models. These results further support the hypothesis that JEV-associated neuronal loss is not only the result of direct viral infection of neurons, but is also related to bystander damage caused by activated microglia.

**Fig. 6.** Cytokine levels after JEV infection. (a) Neuron/glial cultures were mock-infected or infected with JEV, UV-inactivated JEV (JEV/UV) or boiling-inactivated JEV (JEV/boiled) for 48 h. (b) Mixed glial, astrocyte and microglial cultures were mock-infected or infected with JEV for 48 h. The supernatants were collected and subjected to ELISA for the measurement of TNF-α, IL-1β, IL-6 and RANTES. **, P<0.01, n=4 from different batches of preparations.
Primary neuron/glial cultures consist mostly of neurons, astrocytes and microglia. This culture model enables the researcher to study neurotoxicity. The degeneration of neurite processes and loss of the neuronal body after infection revealed the onset of JEV-associated neuronal death in neuron/glial cultures (Figs 1 and 2). Neurotoxicity is hypothesized to develop as a consequence of at least two possibilities. The demise of neurons in neuron cultures after JEV infection indicated that the cause of neurotoxicity was direct viral infection of neurons (Fig. 2a). Mixed glia are composed of astrocytes and microglia. The conditioned medium of JEV-stimulated mixed glia (Fig. 2) and microglia, but not of astrocytes (Fig. 7), was capable of triggering neuronal death, indicating a critical role for the bystander mechanism of neuronal death in JEV-associated neurotoxicity. A previous study by us showed that JEV preferentially infects neurons (~90%), microglia (~80%) and astrocytes (~20–30%) based on immunopositivity of viral antigen. Intriguingly, cultured primary neural cells (neurons, ~10^4 p.f.u. ml^{-1}; astrocytes, ~10^3 p.f.u. ml^{-1}; microglia, ~10^3 p.f.u. ml^{-1}) were not suitable permissive cells compared with BHK21 cells (~10^8 p.f.u. ml^{-1}) for productive replication of JEV (Chen et al., 2004). Despite the low level of infectious virus particles, the severity of neuronal death induced by conditioned medium was similar to direct infection of neurons, indicating that the observed cell death was not the result of the residual JEV released from the infected cells. This hypothesis was further supported by the neurotoxic potential of conditioned medium pre-treated with UV irradiation (Fig. 2), after which the presence of replication-active JEV was minimized. These findings emphasize the importance of both direct killing of neuronal cells by JEV and indirect neuronal death induced by neurotoxic molecules released from JEV-infected microglia in JEV neuropathogenesis. Currently, the nature of the neurotoxin(s) released from JEV-stimulated mixed glia and microglia has not been fully characterized. The ELISA data (Fig. 6), together with data reported in related studies, suggest that the neurotoxicity of mixed glia- and microglia-conditioned medium was primarily the consequence of molecules with neurotoxic actions that were released from the infected cells. TNF-α and IL-1β are potentially neurotoxic molecules in JEV-associated neuronal death, as both neutralizing antibodies attenuated JEV-induced neuronal death (Fig. 8) and both

Fig. 7. Effect of glia-derived conditioned medium on neurotoxicity. The supernatants of mock-infected (Mock CM) and JEV-infected microglia (JEV microglia CM) and JEV-infected astrocyte (JEV astrocyte CM) cultures were collected 48 h after infection and mixed with an equal volume of fresh medium. The conditioned media were added to cultured neurons for 24 h. Neuronal viability was detected by the immunoreactivity of MAP-2. Representative micrographs are shown. Bar, 60 μm.

Fig. 8. Neurotoxic effect of released cytokines. Microglial cultures were mock-infected (Mock CM) or infected with JEV (JEV CM) for 48 h and the supernatants collected. Supernatants isolated from JEV-infected cells were modified by neutralization with control IgG and TNF-α, IL-1β, RANTES and IL-6 neutralizing IgGs (10 μg ml^{-1}) for 30 min before mixing with an equal volume of fresh medium. The modified conditioned media were added to cultured neurons for 24 h. Neuronal viability was detected by the immunoreactivity of MAP-2. The number of viable MAP-2 immunoreactive neurons is depicted. *, P<0.05; **, P<0.01, n=4.
TNF-α and IL-1β have been shown to directly and indirectly injure neurons (Raung et al., 2005, 2007; Ghoshal et al., 2007; Swarup et al., 2007, 2008; Das et al., 2008).

The effective control and ultimate clearance of invading pathogens and removal of infected cells are critical in the host response to infection. Unfortunately, over-reactivity of host responses is also a key event leading to neuropathological changes. The response to cerebral infection with JEV is initiated by the activation of glia with consequent production of pro-inflammatory mediators, and is amplified by the recruitment of peripheral inflammatory cells into the damaged brain. In addition to neuronal damage, clinical and animal studies showed signs of neuroinflammation such as inflammatory cell infiltration/activation and pro-inflammatory mediator production at the lesion sites and in the peripheral circulation (Chaturvedi et al., 1979; Mathur et al., 1988; Khanna et al., 1991; Ravi et al., 1997; Singh et al., 2000; German et al., 2006; Ghoshal et al., 2007). After infection of neuron/glial cultures with JEV, neurotoxicity was accompanied by concomitant activation of glia, as evidenced by morphological transformation (Figs 1 and 2), increased cell proliferation (Fig. 5) and elevated pro-inflammatory mediator production (Fig. 6). These cellular changes and pro-inflammatory responses by JEV infection were similar to those observed in other clinical and experimental studies (Chaturvedi et al., 1979; Mathur et al., 1988; Khanna et al., 1991; Ravi et al., 1997; Singh et al., 2000; German et al., 2006; Ghoshal et al., 2007). The activation of glia and induction of innate immunity appear to initiate and propagate an irreversible immune response leading to neuronal cell death in Japanese encephalitis. Glial activation by viruses has been demonstrated to be triggered by several means, such as direct viral infection, released virus particles, viral proteins, dsRNA, virus replication and neuronal cell death, as well as other immune challenges. Swarup et al. (2008) reported that TNF receptor-associated death signalling mediated neuronal death, contributing to the glial activation and neuroinflammation in Japanese encephalitis. Thus, neuronal injury is a potent physiological trigger of glial activation after JEV infection. In the absence of neuronal cells, direct infection of mixed glia, astrocyte and microglia cultures by JEV caused activation of microglia and astrocytes (Figs 3–6). These findings imply that direct viral infection is an alternative mechanism in triggering glial activation in Japanese encephalitis. Replication-competent JEV infection caused neuronal death (Fig. 1a), induced morphological change (Fig. 4), stimulated cell proliferation (Fig. 5) and elevated pro-inflammatory mediator production (Fig. 6a). Boiling-inactivated JEV failed to cause neuronal death or induce glial activation. Interestingly, UV inactivation blocked all the effects caused by JEV, except for morphological changes (Figs 1 and 4–6). These findings suggest that morphological changes and cell proliferation/cytokine expression in JEV-infected microglia can be triggered by a distinct mechanism. Replication-competent JEV can complete all these alterations. However, replication-incompetent JEV retains its effect on morphological changes, but loses its abilities in other tested activities. The exact mechanisms of action underlying these differences and the potential diversity of morphological changes were not addressed in this study. Phagocytosis-related morphological changes may play a role, as activated and phagocytic microglia were detected in JEV-infected brains (German et al., 2006; Ghoshal et al., 2007) and higher immunopositivity of viral antigen has been detected in JEV-infected microglia by which cells were not recognized as a preferential target for JEV infection (Chen et al., 2004).

Astrocytes and microglia are two essential cells within the nervous system and possess a diversity of biological activities. Astrocytes maintain homeostasis to support the survival and function of neurons through trophic factor release, inflammation modulation and metabolism regulation (Norenberg, 1994; Ridet et al., 1997). Microglia play an important role in immune surveillance, inflammatory processes and tissue regeneration by releasing trophic factors and inflammatory mediators and by phagocytosis (Perry & Gordon, 1988; Gehrmann et al., 1995; Kreutzberg, 1996; Moore & Thanos, 1996). In responding to JEV infection, microglia underwent morphological changes and proliferation, and released biological mediators such as TNF-α, IL-1β, RANTES and IL-6. In contrast, among the cellular and biochemical alterations, JEV-infected astrocytes responded only by releasing RANTES and IL-6 (Figs 3–6). Among the differences observed between microglia and astrocytes, the inability to release TNF-α and IL-1β might partly explain the minor neurotoxic potential of the conditioned medium of JEV-infected astrocytes (Figs 6–8). Neurons, microglia and astrocytes all possess an intrinsic ability to express TNF-α and IL-1β in response to various stimuli in the CNS (Hansisch, 2002). Thus, cells can sense JEV infection and transduce signals to switching molecules in regulating cellular activity. Unlike microglia, JEV-related sensing/signalling cascades were unable to switch on TNF-α and IL-1β induction in astrocytes. Indeed, many studies have demonstrated that astrocytes and microglia showed distinct alterations in response to JEV infection. JEV infection caused the release of TNF-α, IL-1β, IL-6, IL-18 and monocyte chemotactic protein-1 (MCP-1) in microglial cell lines and expression of inducible nitric oxide synthase and cyclooxygenase-2 (Ghoshal et al., 2007; Das et al., 2008). JEV-infected astrocyte cell lines have been shown to express IL-1β, IL-6, IL-8, IL-18, MCP-1 and IP-10 (Das et al., 2008; Mishra et al., 2008). Animal and primary culture studies have revealed that JEV infection elevates expression of several genes in astrocytes, including IL-6, MCP-1, RANTES, IP-10, glial fibrillary acidic protein (GFAP), glutamate aspartate transporter, glutamate transporter-1, nerve growth factor, calyoxphin factor and brain-derived neurotrophic factor (Chen et al., 2000; Bhowmick et al., 2007; Mishra et al., 2007). The discrepancy between astrocytes and microglia in mediating...

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JEV-triggered TNF-α and IL-1β expression may be resolved after completion of promoter reporter assay-related studies. It should be noted that the distinct functional outcome between astrocytes and microglia in this study may be explained partly by differential viral load. Currently, the critical molecules involved in distinct gene expression profiles in microglia and astrocytes following JEV infection have not yet been determined.

In conclusion, following JEV infection, neuronal death is accompanied by concomitant microgliosis and astrogliaosis. Direct killing and bystander damage to neurons contribute to JEV-associated neurotoxicity. In addition to injured neurons, direct viral infection and cytokines are implicated in glial activation, particularly in microglia. Microglia and astrocytes respond to JEV infection by showing distinct alterations such as morphological changes, cell proliferation and pro-inflammatory mediator production. Taken together, our findings further suggest that numerous pro-inflammatory mediators, such as TNF-α and IL-1β, released by JEV-activated microglia rather than by JEV-activated astrocytes, have the ability to amplify the microglial response and cause neuronal death. However, more research is required to elicit the exact causative mechanisms and to identify the determinant switching signalling for the diversity of distinct responses between astrocytes and microglia following JEV infection.

**METHODS**

**Virus infection.** A local Taiwanese strain of JEV, strain NT113 isolated from mosquitoes, was propagated in C6/36 cells utilizing Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS) (Chen et al., 2004). To infect cells, JEV was adsorbed to cultured neural cells at an m.o.i. of 20 for 1 h at 37 °C. After adsorption, unbound virus was removed by gentle washing with PBS. Fresh medium was added to each plate for further incubation at 37 °C.

**Cell cultures.** The protocol for animal studies was approved by the Animal Experimental Committee of Taichung Veterans General Hospital, Taiwan. Cultures of neurons/glia, mixed glia, neurons, microglia and astrocytes were prepared from the cerebral cortices of 1-day-old Sprague-Dawley rats (Chen et al., 2004). In brief, the dissociated cells were plated on poly(D-lysine)-coated (20 μg ml⁻¹) 96-well or 24-well plates or 6 cm dishes at a seeding density of 2.0 × 10⁵ cells per well, 1.0 × 10⁵ cells per well or 2.0 × 10⁶ cells per dish, respectively. One day after seeding, the culture medium was replaced with minimum essential medium supplemented with 10% FBS and 10% horse serum. After 4 days, the medium was changed and replaced with fresh serum-containing medium. For cortical neurons, the culture medium was replaced with neurobasal medium supplemented with B27. Cytosine arabinoside (10 μM) was added to the medium on days 3 and 4 to inhibit non-neuronal cell division. These neuron/glia and neuron cultures were used for experiments after 10–12 days of culture. For mixed glia, the cell pellets were resuspended in DMEM/F-12 Nutrient Mixture (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. The medium was replenished 4 days after plating and changed every 3 days. The resultant mixed glial cultures were used 14–16 days after plating. Astrocyte and microglial cultures were separated by shaking mixed glial cultures at a speed of 200 r.p.m. for 24 h. The retained astrocytes and detached microglia were replated and maintained in DMEM/F12 containing 10% FBS. Cell composition was identified and estimated by immunocytochemistry using antibodies against MAP-2 for neurons (Transduction Laboratories), GFAP for astrocytes (Santa Cruz Biotechnology) and CD68 for microglia (BioSource). In general, neuron/glial cultures consisted of 30–40% neurons, 40–45% astrocytes and 10–15% microglia. Mixed glial cultures contained ~85% astrocytes and ~15% microglia. The purities of the neurons, astrocytes and microglia were all greater than 95%.

**Conditioned medium treatment.** Mixed glial, astrocyte and microglial cultures were mock-infected or infected with JEV for 48 h. The supernatants were collected and mixed with an equal volume of fresh DMEM/F12 containing 1% FBS, referred to as conditioned medium in this study. The conditioned medium was modified by UV exposure (254 nm exposure for 30 min), boiling (94 °C incubation for 15 min) or the addition of neutralizing antibody for 30 min (control, TNF-α, IL-1β, RANTES or IL-6 IgG at 10 μg ml⁻¹).

**Immunocytochemical staining.** The tested cells (in 24-well plates) were washed twice with PBS, fixed with 4% paraformaldehyde in phosphate buffer (0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄) for 10 min, permeabilized with 0.1% Triton X-100 for 15 min and washed with PBS. The cells were blocked with 5% skimmed milk in PBS for 30 min. The cells were then incubated with primary antibody overnight at 4 °C, followed by washing with PBS. After washing, the cells were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, the colour was developed with 3,3′-diaminobenzidine and observed by light microscopy.

**Microscopic analysis.** A visible cell body and a length of neurite process greater than two cell bodies defined MAP-2-positive neurons. For the quantification of neurons, the data were expressed as number of cells per 0.5 mm². Three fields with a surface area of 0.5 mm² were randomly selected and counted in a well of a 24-well plate. Microglia were characterized by their process-bearing morphology and rounded, darkly stained cell morphology in neuron/glial and mixed glial cultures. Microglia possessing a small, rounded cell body (~25 μm in diameter) without a process-bearing morphology and cytoplasmic expansion (more than twice the cell volume) and branches were noted in the microglial cultures. For the quantification of microglia, the total number of CD68-positive cells was counted in a well of a 24-well plate. Four replicates were conducted for each experiment. Complete experiments were carried out using three different batches of preparations.

**BrdU incorporation.** Cell proliferation and cellular DNA synthesis were evaluated by assessing the levels of BrdU incorporation, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Briefly, cells were labelled with BrdU (10 μM) for 24 h after JEV infection. After fixation and permeabilization, cells were incubated with horseradish peroxidase-labelled BrdU antibody at room temperature for 1 h. The immune complexes were detected by a subsequent 3,3′,5,5′-tetramethylbenzidine reaction, and the absorbance of the resultant colour was read at 450 nm in a microtitre plate spectrophotometer.

**ELISA.** The levels of TNF-α, IL-1β, IL-6 and RANTES in the supernatants were measured using an ELISA kit according to the manufacturer’s instructions (R&D Systems).

**Statistical analysis.** Data are expressed as mean values ± SD. Statistical analysis was carried out using one-way analysis of variance, followed by Dunnett’s test to assess the statistical significance between treated and untreated groups in all experiments. A level of P<0.05 was considered statistically significant.
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