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The blood-brain barrier in the cerebrum is the initial site for the Japanese encephalitis virus entering the central nervous system

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Japanese encephalitis (JE) virus is a member of the encephalitic flaviviruses and frequently causes neurological sequelae in a proportion of patients who survive the acute phase of the infection. In the present study, we molecularly identified viral infection in the brain of mice with rigidity of hindlimbs and/or abnormal gait, in which JE virus particles appeared within membrane-bound vacuoles of neurons throughout the central nervous system. Deformation of tight junctions (TJs) shown as dissociation of endothelial cells in capillaries, implying that the integrity of the blood-brain barrier (BBB) has been compromised by JE virus infection. BBB permeability evidently increased in the cerebrum, but not in the cerebellum, of JE virus-infected mice intravenously injected with the tracer of Evans blue dye. This suggests that the permeability of the BBB differentially changed in response to viral infection, leading to the entry of JE virions and/or putatively infected leukocytes from the periphery to the cerebrum as the initial site of infection in the central nervous system (CNS). Theoretically, the virus spread to the cerebellum soon after the cerebrum became infected. *Journal of NeuroVirology* (2008) **14,** 514–521.

Keywords: BBB impairment; differential disruption; initial site for infection; Japanese encephalitis virus; tight junction

Introduction

Japanese encephalitis (JE) virus is one of about 70 members of the genus *Flavivirus* in the family Flaviviridae; its genome is composed of single-stranded, positive-sense RNA of approximately ~11,000 nucleotides in length (Chambers *et al*, 1990; Lindenbach *et al*, 2001). JE virus is mainly amplified in pigs and birds and is primarily transmitted by *Culex* mosquitoes between vertebrates (Detels *et al*, 1970; Hsu *et al*, 1978), causing a significant number of cases of human encephalitis in the world, particularly Asian and south Pacific regions (Endy and Nisalak, 2002). Most JE patients present typical clinical symptoms including headaches, vomiting, an altered mental state, as well as dystonia, rigidity, and a characteristic mask-like facies (Chuang *et al*, 2002). Clinical sequelae including mental retardation and/or movement disorders may appear in patients who survive the disease (Huang *et al*, 1996; Whitley and Gnann, 2002).

Magnetic resonance imaging (MRI) findings have revealed that JE virus infection results in a characteristic pattern of mixed intensity or hypodense lesions in the thalamus and also in the basal ganglia and midbrain (Kalita and Misra, 2000). Involvement of the brain stem and deep nuclei may be responsible for the frequent occurrence of acute respiratory failure and death, and that of the basal ganglia and thalamus for the frequent movement disorders of JE patients (Johnson, 1987; Huang *et al*, 1997; Kalita and Misra, 2000). It has been estimated that there is about 30% case fatality rate with JE virus infection in general population. Sequelae including motor weakness,

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intellectual impairment, and seizure disorders have been reported to appear in 15% ~50% of survivors (Misra and Kalita, 1997; Whitley and Gnann, 2002).

After the bite of an infected mosquito, the JE virus is thought to amplify in dermal tissues and then lymph nodes via migration of dendritic (Langerhans) cells before invading the central nervous system (CNS) (Johnson *et al*, 2000; Solomon *et al*, 2000). Flaviviruses such as Murray Valley encephalitis virus, St. Louis encephalitis virus, and JE virus have been speculated to enter the CNS via the olfactory pathway (Monath *et al*, 1983), transcytosis across cerebral capillary endothelial cells (Liou and Hsu, 1998), or virion budding on the parenchymal side after replication in endothelial cells (McMinn *et al*, 1996). Nevertheless, many infections of encephalitic flaviviruses are diffusely spread throughout the brain, revealing that the hematogenous route is probably more important in mediating virus particle entry into the CNS (Mims, 1957; Johnson *et al*, 1985). In this study, a mouse model with intravenous inoculation was used to evaluate the effect of JE viral infection on the integrity of the blood brain barrier (BBB) and its association with virus entry into the brain parenchyma from peripheral blood.

Results

Infection of the brain by the JE virus

Eight of 39 mice infected with the JE virus appeared clinical signs with rigidity of the hindlimbs and an abnormal gait 5 ~7 days after inoculation, while none of 10 mice inoculated with diluted culture medium showed related symptoms (Table 1). All mice with symptoms, and some without symptoms collected at day 4 ~7 post-inoculation, were demonstrated to be infected by JE virus in both the cerebrum and cerebellum (Table 1). According to the results of an RT-PCR that amplified a 291-bp fragment of DNA from both the cerebrum and cerebellum of infected mice, the corresponding DNA fragment was not detected in any control mouse (Figure 1). In addition, 6 of 14 mice containing viral DNA in the CNS showed no clinical signs, indicating a high level of subclinical infection even they are infected through intravascular inoculation (Table 1). At the ultrastructural level, the JE virus extensively existed in hypertrophic vacuoles of ER throughout the brain of the inoculated, but not the control, mice. In specific, JE virions in a round shape with prominent envelope were seen in moribund neurons (with scarce organelles in the cytoplasm) in the cerebrum (Figure 2A) as well as astrocytes in the cerebellum (Figure 2B).

Changes in tight junction (TJ) integrity and PBMC penetration

Occludin, a component protein of TJs in the BBB, normally showed deep staining with specific antibodies in the healthy brain (Figure 3A), while the protein disappeared from the brain of mice with evident neurological symptoms (Figure 3B) in cross-sections of capillaries. A breakdown of the BBB, shown as a dissociated gap between endothelial cells of the capillary at ultrastructural level, was evidenced in the brain of three infected mice (Figure 4A). In contrast, the tight junction remained intact in the BBB of healthy mice (Figure 4B). Leukocytes, likely neutrophils, were shown to penetrate the capillary wall in the inflamed area indicated by severe edema based on a semi-thin section from the brain of a sick mice (Figure 5A); which was also shown in an ultra-thin section of brain tissue from the same mice (Figure 5B). Perivascular cuffing with a number of penetrated leukocytes around capillaries was frequently observed in the brain of sick mice as presented in an ultra-thin section of the brain tissue (Figure 5C).

Differential changes in BBB permeability

Whole brains taken from 10 JE virus-inoculated ICR mice were stained consistently when the mice were injected with Evans blue before dissection. The staining intensity was revealed to be time-dependent. No sign of positive staining was shown in any one of 4 mice inoculated with PBS or EMEM culture fluid (Figure 6A). In contrast, a light blue color appeared in the cerebrum of a mouse (with symptoms of light paralysis) 7 days post-inoculation (Figure 6B), while extensive deep blue lesions were seen in that of a paralytic mouse (with eye hemorrhaging) 10 days post-inoculation (Figure 6C). The cerebellum of inoculated mice was not or was rather lightly stained according to observations in this study (Figure 6B, C).

Table 1 Status of sickness and viral infection in mice with intravenous inoculation of Japanese encephalitis (JE) virus suspension*

		2	3	4	5	6	7	
days post-inoculation		no. positive/no. inoculated						Total
sickness		0/3	0/6	0/6	1/6	3/6	4/6	8/39
virus infection	cerebrum	0/3	0/6	1/6	4/6	5/6	4/6	14/33
	cerebellum	0/3	0/6	1/6	4/6	4/5	4/6	13/32

*A total of 39 ICR strain mice (4 ~5 weeks old) were inoculated with the CJN strain of the JE virus (1 x 10^6 pfu/mouse). After inoculation, 3 ~6 mice were sacrificed daily from 2 to 7 days. Virus infection (no. positive/no. detected) in either cerebrum or cerebellum was confirmed based on the result of RT-PCR to amplify a 291 bp DNA fragment (see Figure 1).

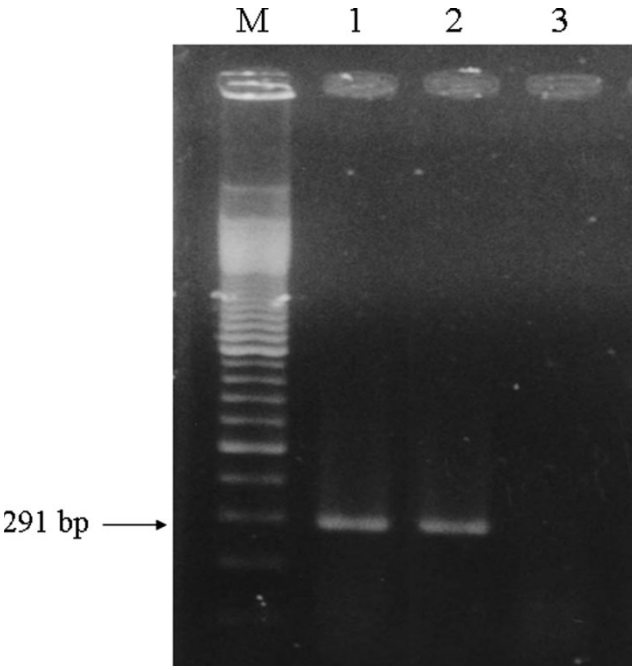

Figure 1 A 291-bp gene fragment of the envelope protein amplified by RT-PCR from Japanese encephalitis (JE) virus in the infected brain tissues among 39 inoculated and but not in 10 control mice. Examples with positive results have been shown in this figure, usually appearing in mice from 4 days after inoculation. M, 100-bp marker; lane 1, JE virus-infected cerebrum; lane 2, JE virus-infected cerebellum; lane 3, negative control from the brain tissue of a mouse inoculated with virus-free medium.

Discussion

Japanese encephalitis remains a major cause of viral encephalitic disease in the Asian and Pacific regions (Solomon, 2003). However, little is known about the pathogenesis, particularly the mechanism of viral spread from the periphery to the CNS (Myint *et al*, 2007). A variety of flaviviruses were speculated to enter the CNS via the olfactory pathway (Monath *et al*, 1983) or across cerebral capillary endothelial cells (Liou and Hsu, 1998). Nevertheless, features including the diffuse infection throughout the brain and no viral antigens in the choroid plexus or ependyma indicate that viruses probably enter the CNS via a hematogenous route (Kimura-Kuroda *et al*, 1992), especially when severe viremia is induced (Yamada *et al*, 2004). JE virus has repeatedly been demonstrated to infect neurons in the brains of experimental animals and humans (Johnson *et al*, 1985; Yamada *et al*, 2004) and is usually not cleared from tissues by elicited antibodies (Desai *et al*, 1995). It is known that encephalitic flaviviruses induce apoptosis in infected neurons and subsequently cause fatal outcomes to the host (Despres *et al*, 1998; Xia *et al*, 2001). The present study has indeed revealed extensive infection of neurons, resulting in cellular defects, in the cerebrum as well as cerebellum.

Cerebral and cerebellar capillary endothelial cells are responsible for the BBB (Silwedel and Förster, 2006). In both animals and humans, the BBB generally prevents viral invasion into the CNS (Ballabh

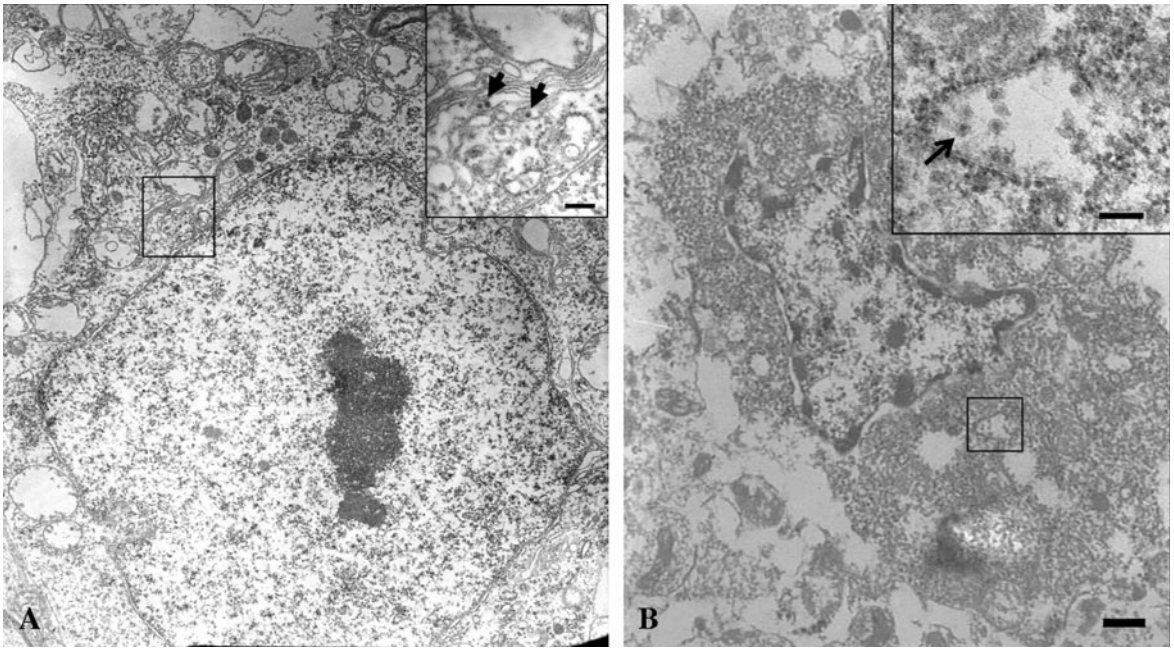

Figure 2 Electron micrographs illustrating Japanese encephalitis (JE) virus infection in a moribund neuron (Neu) of the cerebrum. The inset is a magnified photograph of the rectangular area where JE virions can be seen in the hypertrophic endoplasmic reticula (ER). Scale bar, 1 μm. Scale bar for the inset, 200 nm. (B) Electron micrograph showing the JE virus infecting an astrocyte in the mouse cerebellum, particularly in hypertrophic ER. Scale bar, 500 nm. The inset of a magnification of the rectangular area showed that mature JE virions (arrow) were assembled in the hypertrophic ER. Scale bar, 100 nm.

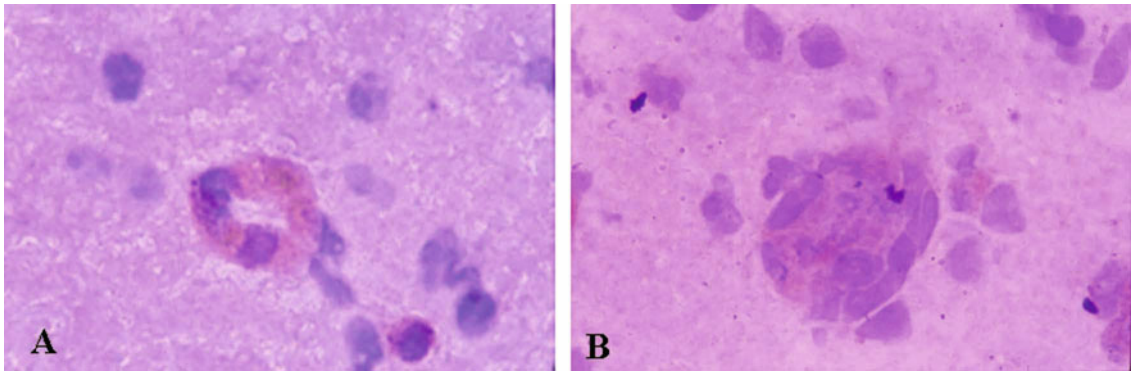

Figure 3 Immunohistochemical detection of occludin that disappeared from tight junctions of capillaries in a Japanese encephalitis virus-infected mouse. (**A**) A cross-section of a capillary with deep-brown staining in/between endothelial cells from an uninfected mouse brain. (**B**) Deep-brown staining absent from the cross-section of a capillary from an infected mouse brain (10 days post-inoculation). Pictures were taken at 1000x magnification.

et al, 2004), unless it has been disrupted, resulting in increased permeability and inflammatory cell infiltration (Bell *et al*, 1993; Muller *et al*, 2005). Thus JE viruses may gain entrance into the CNS through the breach that is created in the barrier at the time of viral transmission (Hase *et al*, 1990). The BBB integrity was evidently weakened in the primary phase of virus infection when monitored via an injection of Evans blue. This feature can be marked by the modified distribution of occludin, a TJ component protein which aggregates on the surfaces of endothelial cells and essentially brings the cells very close together (Traweger *et al*, 2002). TJs are present between cerebral endothelial cells and form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain (Ballabh *et al*, 2004). As a result, displacement or disappearance of occludin in TJs may be referred to as a feature of alteration in the BBB integrity, leading to increased permeability of the BBB (Bolton *et al*, 1998). It is now known that viral replication in peripheral tissues may trigger a Toll-like receptor inflammatory response that alters the blood-brain barrier (Wang *et al*, 2004). The stimulus capable of causing changes of the BBB permeability was also reported to be derived from effects of the chemokine monocyte chemoattractant protein (MCP-1) (Yamada *et al*, 2004; Stamatovic *et al*, 2005).

Various stimuli are able to cause leukocyte migration into the CNS (Ransohoff *et al*, 2003). The BBB with increased permeability actually allows PBMCs to migrate from the peripheral blood into brain

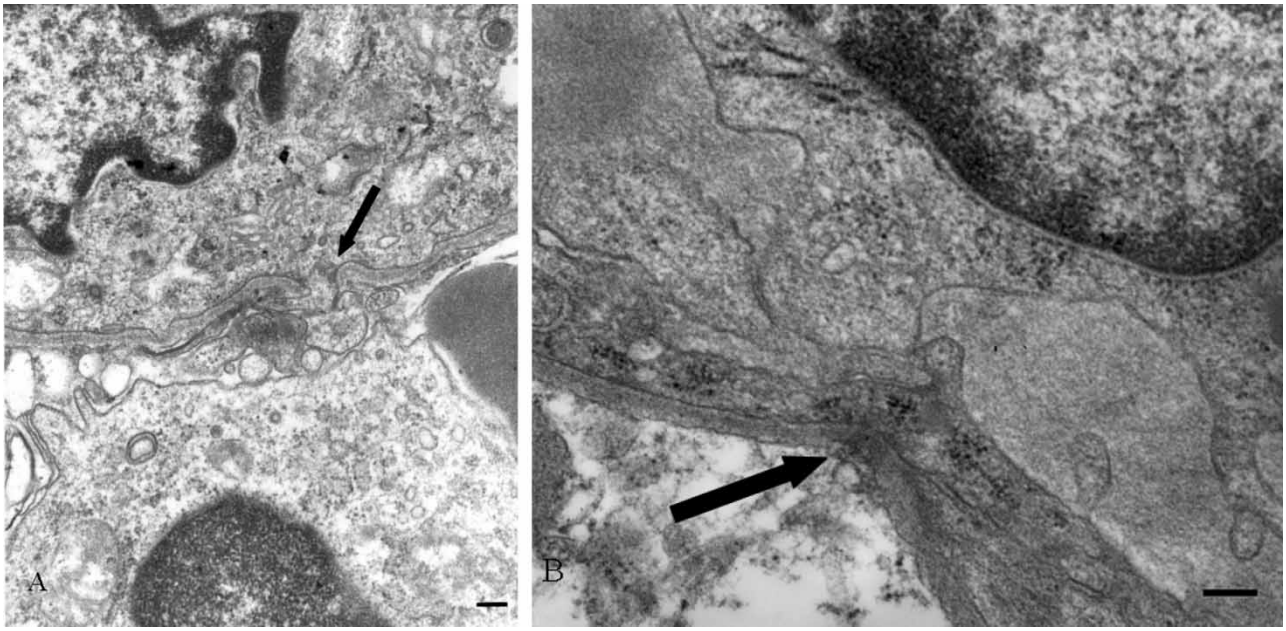

Figure 4 Electron micrograph of blood-brain barrier (BBB) breakdown. Dissociation of the tight junction (arrow) was shown in the cerebrum of a mouse infected by the Japanese encephalitis virus for 7 days. (B) The tight junction (arrow) remained intact in the BBB of a control mouse. Scale bar for (A) and (B), 200 nm.

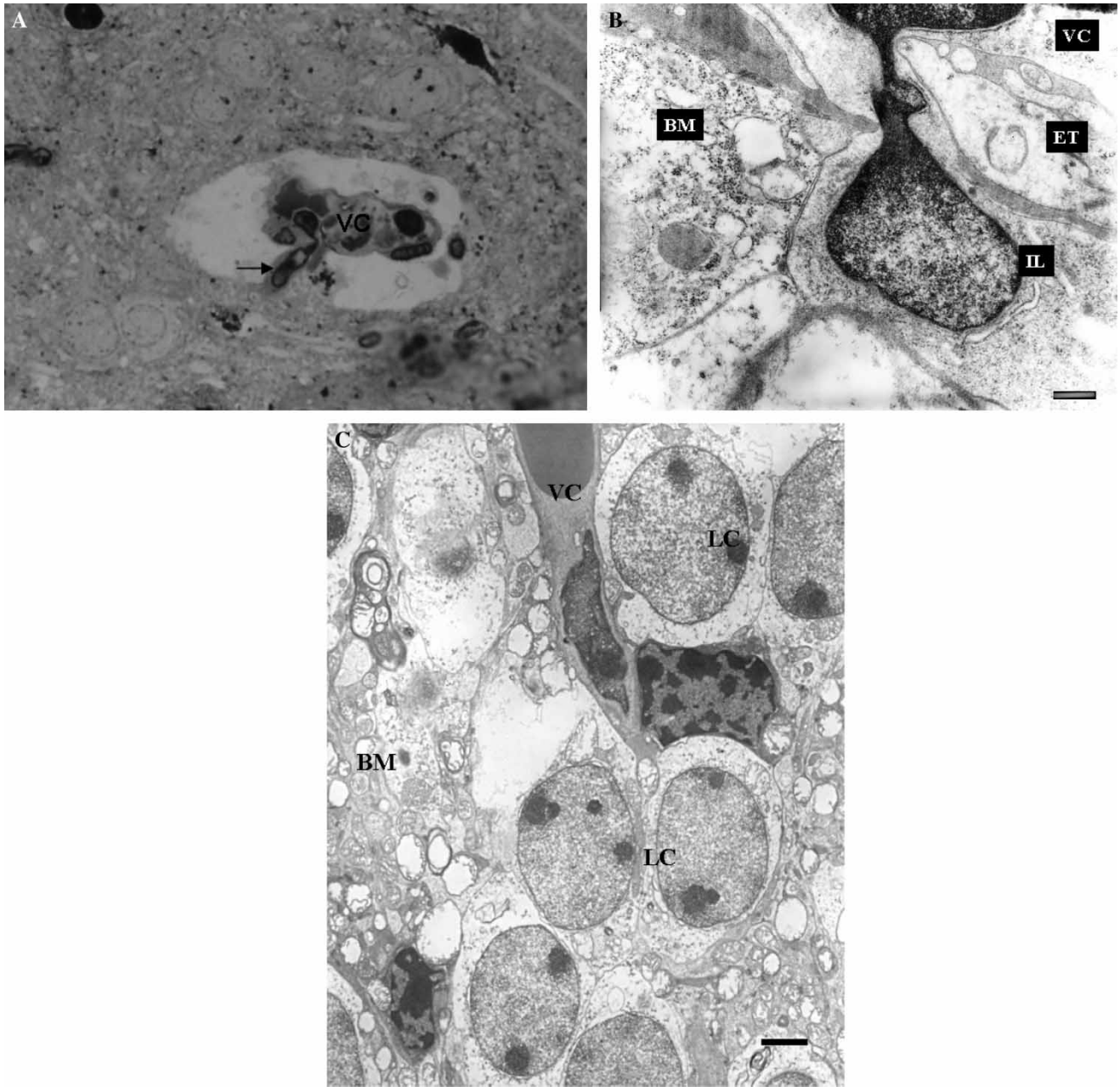

Figure 5 A neutrophil (arrow) penetrating the brain parenchyma through the disrupted tight junction of the capillary shown on a 1-μm semi-thin section (**A**) and an ultrastructural section (**B**). Perivascular cuffing with a number of penetrated leukocytes (arrow) around the capillary appeared on an ultra-thin section of the brain tissue from the sick mouse (**C**). BM, brain matrix; ET, endothelium; IL, invading leukocyte; LC, invaded leukocyte; VC, blood vessel cavity. Original magnification of (**A**), 1000x; scale bar for (**B**), 500 nm and (C), 2 μm.

tissues (Stephens *et al*, 2003; Diamond and Klein, 2004). Normally, lymphocytes enter the CNS constantly in small numbers (Hickey *et al*, 1991), but their presence in the CNS may increase in response to viral infections (Griffin *et al*, 1992). We have detected peripheral PBMCs isolated from inoculated mice infected by the JE virus as early as 3 days after inoculation (Chuang *et al*, 2003). More importantly, the present study frequently shows that leukocytes move across endothelial cells of capillaries at the site where TJs appear to be dissociated. This suggests that at least some inflammatory leukocytes that became infected peripherally move along with the current of virion migration to CNS tissues (McMinn *et al*, 1996; Chuang *et al*, 2003). Thus far, observation on migrating leukocytes with viral infection remains to be worked out at the ultrastructural level.

In an animal model of multiple sclerosis (MS), the cerebellar BBB was shown to be especially vulnerable at the beginning of increased permeability in the cerebellum (Silwedel and Förster, 2006). However, the BBB permeability was shown

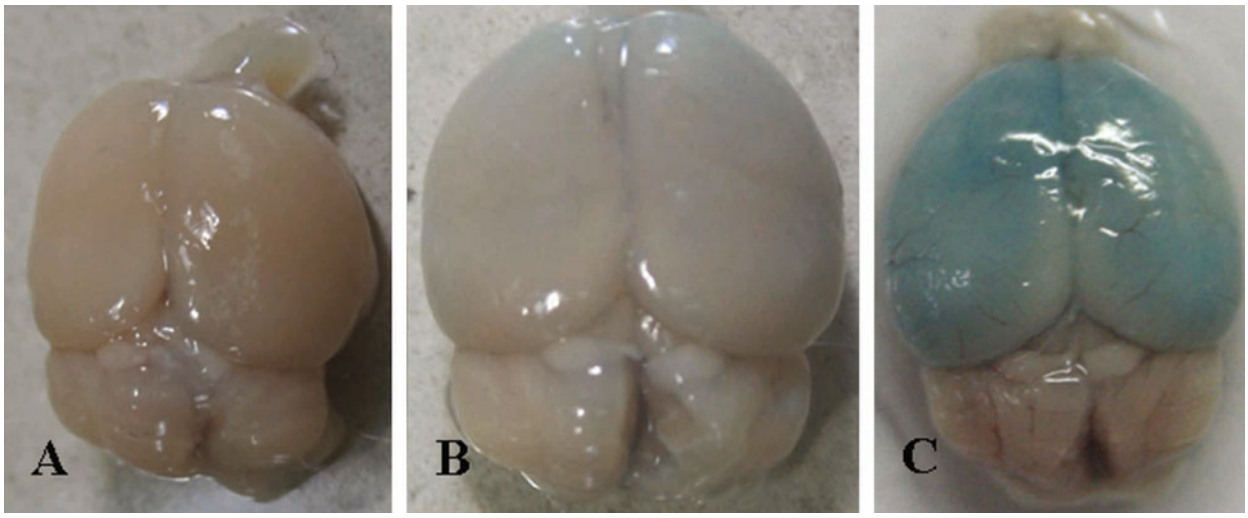

Figure 6 Whole brain dissected from a Japanese encephalitis (JE) virus-inoculated ICR mouse that was then injected with Evans blue solution. (**A**) The mouse (without symptoms) inoculated with PBS and dissected 7 days post-inoculation. (**B**) The mouse (with light hindlimb paralysis) inoculated with a JE virus suspension (1×10^6 PFU/mouse) and dissected 7 days post-inoculation. **C:** A mouse (with severe hindlimb paralysis and eye hemorrhages) inoculated with a JE virus suspension (1×10^6 PFU/mouse) and dissected 10 days post-inoculation. Original magnification, 60 ×.

to evidently increase in the cerebrum, but not in the cerebellum, in JE virus-infected mice intravenously injected with Evans blue dye as the tracer. This suggests that differential changes in barrier properties of cerebral and cerebellar brain microvascular endothelial cells may occur, probably depending on the involved inflammatory stimuli (Silwedel and Föorster, 2006). Herein, we propose that inflammatory leukocytes with viral infection pass through dissociated TJs in the cerebral BBB as a way to enter the CNS in order to lyse JE virus-infected neuronal cells or glial cells such astrocytes (Konishi et al, 1991). In the present study, astrocytes which actually serve as a component of the BBB and the first point to confront the penetrated virions or leukocytes are frequently seen to be infected in various areas of the brain tissue. In conclusion, the severely impaired BBB in the cerebrum may play as the initial site for virus entry, usually occurring as early as 4 days post-inoculation. It is followed by a rapid spread of viral progeny produced in the cerebrum which contains numerous moribund infected neurons to the cerebellum by crossing over the transverse fissure as viral infection can be detected by RT-PCR at the same day in both parts of the brain.

Materials and Methods

Virus and cell culture
The T1P1 strain of the JE virus, isolated from the mosquito, *Armigeres subalbatus* (Chen *et al*, 2000), was propagated in C6/36 cells derived from the mosquito *Aedes albopictus*, and virus titers were determined using baby hamster kidney (BHK-21) cells. Both types of cells were maintained in Eagle's minimum essential medium (EMEM; GIBCO®, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) as described earlier (Chiou and Chen, 2001). C6/36 cells were incubated in a closed system at 28°C, whereas BHK-21 cells were incubated at 37°C in a 5% CO_2 atmosphere.

Virus titration
The virus was titrated by means of plaque assays on BHK-21 cells following the description in one of our previous reports (Chiou and Chen, 2001). Calculation of virus titers was based on the number of formed plaques, which was expressed as plaque forming units per ml (PFU/ml).

Inoculation of JE virus in mice
Female ICR outbred mice (6 ~ 8 weeks old) were used in this study. Among these, 39 mice were intravenously injected with a dosage of 1×10^6 PFU/mouse of JE virus suspension diluted with phosphate-buffered saline (PBS, pH 7.4) to a final volume of 100 μl. The other 10 were inoculated with a virus-free solution diluted from the cell culture medium; these were used as the control to confirm the infection of mice inoculated with virus suspension. The movement and body coordination of inoculated mice were monitored daily for at least 1 week. Mice with evident symptoms (movement disorders, mostly rigidity of the hind-limbs) were sacrificed to harvest tissues of the brain for further studies.

Reverse-transcriptase polymerase chain reaction (RT-PCR)
Viral RNA was extracted from tissues of the brain following a protocol described in the QIAamp

viral RNA handbook (QIAGEN®, Hilden, Germany). Primers used for the subsequent RT-PCR included the complementary primer (5′-GTTCCATCTCGAC CAGCAC-3′) and the sense primer (5′-AGTTAACAT CAGGCCACCTGA-3′) under conditions defined previously (Chiou and Chen, 2001). The PCR product was a 291-bp fragment of the envelope (E) protein of the JE virus, that was analyzed by immediately running electrophoresis on a 2% (w/v) agarose gel containing 10 μl ethidium bromide (1 mg/ml in RNase-free water). The band patterns on the gel were visualized and photographed using a Polaroid camera on a UV-illuminated board.

Evans blue staining

ICR mice were used in this study for inoculation with a JE virus suspension at the dose of 1×106 PFU/mouse. Ten symptomatic and 4 control mice sampled at 7 or 10 days post-inoculation were injected with 100 μl of Evans blue solution (2% in PBS) intravenously (Chen *et al*, 2004). After 1 h, all injected mice were anesthetized with 35% chloral hydrate carried out by intracardial perfusion of PBS at a speed of 9 ml/15 sec for 5 min. Whole mouse brains were subsequently dissected out for photography to localize the distribution of stained areas.

Frozen-sectioning

Tissues of the brain embedded in tissue-freezing medium® (Jung, Nussloch, Germany) were transiently frozen in liquid nitrogen and then cut with a cryomicrotome (CM3050S; Leica, Mannheim, Germany). The collected $7 \sim 8$-μm-thick sections were placed on slides coated with Silane S (Muto Pure Chemicals, Tokyo, Japan), and then fixed in cold acetone for 15 min before further use.

Electron microscopy

For electron microscopy, brain tissues were dissected out from healthy or infected mice, and immediately fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. After the tissues were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer for 2 h at room temperature, they were washed with 0.2 M cacodylate buffer 3 times. Again, tissues were washed with 0.2 M cacodylate buffer 3 times and then dehydrated through an ascending graded series of ethanol. Tissues were finally embedded in Spurr's resin (Electron Microscopy Science, Hatfield, PA, USA) and polymerized at 70°C for 72 h. The trimmed tissue blocks were sectioned with an ultramicrotome (Reichert Ultracut R, Leica, Austria). Semi-thin (1-μm-thick) sections were stained with 1% toluidine blue and observed under a compound microscope, while ultra-thin sections were sequentially stained with saturated uranyl acetate in 50% ethanol and 0.08% lead citrate. Selected images were observed and photographed under an electron microscope (JEOL JEM-1230, Tokyo, Japan) at 100 kV.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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