Early Mortality Following Intracerebral Infection with the Oshima Strain of Tick-Borne Encephalitis Virus in a Mouse Model

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ABSTRACT. Tick-borne encephalitis virus (TBEV) is a zoonotic agent that causes acute central nervous system (CNS) disease in humans. In this study, we examined the pathogenic process following intracerebral infection with the Oshima strain of TBEV in a mouse model. Intracerebral infection resulted in dose-dependent mortality, and all mice died following challenge with 10^2 PFU or more of the virus within 10 days. Acutely necrotic neurons and widespread inflammation were observed throughout the CNS. We therefore conclude that mortality following intracerebral infection results from a direct CNS pathology.
KEY WORDS: central nervous system, mouse, pathogenesis, Tick-borne disease, virus infection.

Tick-borne encephalitis virus (TBEV), belonging to the genus *Flavivirus* in the family *Flaviviridae*, is a zoonotic agent of acute central nervous system (CNS) disease in humans [4, 12]. TBEV is transmitted by *Ixodes* tick species and rodents in nature and infects humans through the bite of an infected tick [12]. TBEV is geographically and genetically divided into three subtypes comprising the European, Siberian and far eastern subtypes [5, 8]. Our previous data showed that TBEV is also distributed throughout southern parts of Hokkaido, Japan [23–25].

In human cases, the neurological symptoms include fever, headache, meningitis, meningoencephalitis and meningoencephalomyelitis, the latter being observed in the most severe cases [4]. When death follows, it is usually within 5 to 7 days of the onset of neurological signs. The pathological findings in the brain in human cases are nonspecific, and lesions containing TBEV antigens are located in the brain stem, cerebrum, cerebellar cortex, pons, cerebellum, thalamus and motor neurons [4, 6, 7]. Thus, the clinical features are not unique to TBE, and laboratory diagnosis is required to distinguish it from other neurological disorders [1, 10, 14].

CNS pathology following TBEV infection is the consequence of viral infection of the corresponding cells and the resulting inflammatory responses in the CNS. Direct viral infection of neurons is considered to be the major cause of neurological disease because viral infections cause apoptosis or degeneration of neurons *in vivo* and *in vitro* [3, 11, 18, 21]. In addition, recent studies have demonstrated that immunopathological effects also contribute to the severity of CNS pathology [19, 27].

The laboratory mouse model is the system most commonly employed to study the CNS pathology of TBEV *in vivo* [2, 17, 22, 26]. The CNS pathology of TBEV consists of the two distinct features of neuroinvasiveness and neurovirulence, and death has been used as an index of pathogenesis [13, 15]. Thus, mortality following peripheral infection is considered to represent neuroinvasiveness, whereas mortality following direct intracerebral infection represents neurovirulence [13].

However, our previous studies in a mouse model found that peripheral infection with the Oshima strain of TBEV caused a dose-independent mortality [2, 9]. Furthermore, we showed that following peripheral infection mice died either early or late and that mortality resulted from a combination of CNS pathology, systemic stress and inflammatory responses [9]. Together, these results indicate that peripheral infection with TBEV does not represent neuroinvasiveness alone.

On the other hand, it is considered that the patterns and the mechanism of mortality following intracerebral infection differ from those of peripheral infection. Thus, in this study we investigated the pathogenic mechanisms that correlate with fatal infection following intracerebral infection with the Oshima strain of TBEV in a mouse model.

MATERIALS AND METHODS

Virus and cells: The stock virus of the Oshima strain of TBEV [23] was prepared in baby hamster kidney (BHK) cells after a few passages through suckling mouse brains. The BHK cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 8% fetal calf serum (FCS). All

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experiments using live TBEV were performed in a biosafety level 3 laboratory of the Tokyo Metropolitan Institute for Neuroscience, according to standard BSL3 guidelines.

Mice: Five-week old female C57BL/6j (B6) mice (Japan SLC, Inc., Hamamatsu, Japan) were anesthetized and then intracerebrally inoculated with a range of 10^{-1}–10^6 PFU of TBEV diluted in EMEM containing 2% FCS. Mock-infected mice were inoculated with EMEM from supernatants of BHK cells. Mice were weighed daily and observed for clinical signs such as paralysis. Morbidity was determined by the degree of continuous weight loss, as indicated by a weight ratio of below 1.00 of compared with day 0. The experimental protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience.

Virus titrations: Three mice inoculated with 10^5 PFU of virus were sacrificed, and the bloods, lungs, thymuses, spleens, brains and spinal cords were removed after perfusion with cold phosphate-buffered saline (PBS) at 3 and 6 days post-infection (pi). The brains were divided into four parts: brain cortex, thalamus, cerebellum and brain stem. Tissues were kept frozen at –80°C until use. Each tissue type was homogenized in ten volumes of PBS containing 10% FCS and diluted with EMEM containing 2% FCS. Virus titers were determined by plaque-forming assays using BHK cells and were calculated as PFU/g of tissue [20].

Histopathological examinations: Mock-infected mice and mice infected with 10^5 PFU of TBEV were anesthetized and perfused with 10% phosphate-buffered formalin 8 days pi. Fixed tissues of thymus, lung, liver, kidney, spleen, small intestine, brain, spinal cord and maxilla including nasal cavity were routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical detection of the TBEV antigens was performed as described previously [16]. Rabbit polyclonal antibody against anti-E protein was used to detect TBEV antigens [28].

ELISA: Serum samples were collected from 3 mock-infected mice and 3 mice infected with 10^5 PFU doses at 3 and 6 days. The levels of corticosterone and tumor necrosis factor-alpha (TNF-α) in the serum were measured using competitive EIA and sandwich ELISA kits for corticosterone (Assaypro, St. Charles, MO, U.S.A.) and TNF-α (Endogen, Woburn, MA, U.S.A.) according to the manufacturer's instructions.

Statistical analysis: Analysis of variance and the Student's *t*-test were used to assess the significance of differences in the degree of weight changes, viral loads, the numbers of leukocytes, and the expression levels of cytokines in brains and sera. A *P* value of <0.05 was considered statistically significant.

RESULTS

Mortality and morbidity: Following intracerebral infection of groups of B6 mice with sequentially increasing
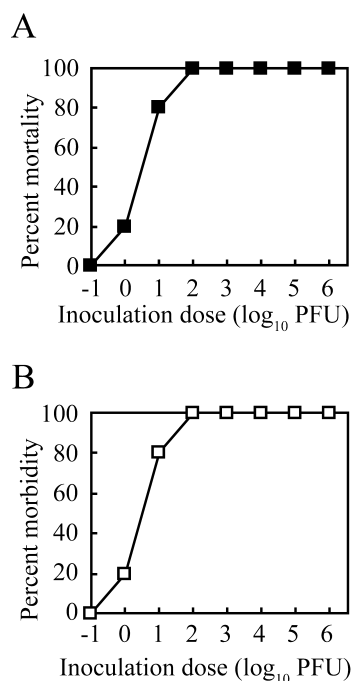

Fig. 1. Mortality and morbidity following intracerebral infection with the Oshima strain of TBEV in B6 mice. Five mice in each group were intracerebrally infected with increasing concentrations of virus ranging from 10^{-1} to 10^6 PFU. (A) Mortality rates were recorded for 21 days. (B) Morbidity of mice was estimated by the degree of weight loss after 21 days.

doses, mice exhibited a dose-dependent curve of mortality, and infection with more than 10^2 PFU resulted in 100% mortality (Fig. 1A). Mice began to die at 7 days pi, and most died within 10 days pi (Fig. 2A). The mean survival times were 8.8 ± 1.89 days (10^6 PFU), 7.8 ± 0.51 days (10^5 PFU), 8.0 ± 0.00 days (10^4 PFU), 10.2 ± 2.49 days (10^3 PFU) and 9.2 ± 1.88 days (10^2 PFU), with no significant differences between the challenge doses. These observations indicate that intracerebral infection induces early death in mice even after low dose challenge.

TBEV-infected mice remained asymptomatic for 4 to 5 days and then exhibited clinical signs including weight loss, slowness in movement, ataxia, piloerection and anorexia. Mice exhibited neurological signs of paralysis such as rigidity and flaccid paralysis from 7 days pi, although 33% of the dead mice did not exhibit apparent paralysis before death.

Body weight loss was the first clinical observation. Thus, we estimated the onset of disease by whether or not the weight of each mouse decreased compared with control uninfected mice. The onset of weight loss occurred at 4.8 ± 0.51 days pi (10^6 PFU), 4.8 ± 0.51 days pi (10^5 PFU), 4.4 ± 0.62 days pi (10^4 PFU), 5.6 ± 1.02 days pi (10^3 PFU) and 6.6 ± 1.31 days pi (10^2 PFU), with no significant differences
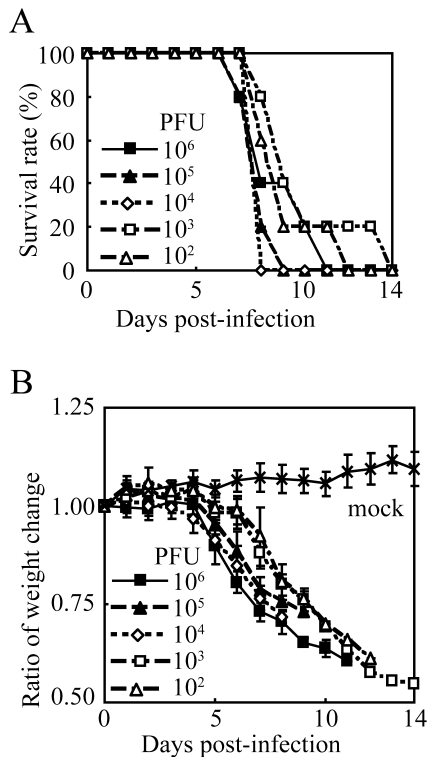
Fig. 2. Survival curves and weight changes following intracerebral infection with 10^2 to 10^6 PFU of the Oshima strain of TBEV in B6 mice (five mice in each group). (A) Survival curves were recorded for 14 days. (B) Average daily weight changes of mice infected with 10^2 to 10^6 PFU and mock-infected mice. Daily weight loss compared with weight on day 0 is represented as a ratio. Error bars represent the standard deviations (n=5).

acute viral replication throughout the CNS, and thus the early death observed following intracerebral infection was directly related to the severity of viral infection in the CNS.

Histopathological features of mice: We next examined the histopathological features following intracerebral infection. Corresponding to the viral load, acutely necrotic neurons and mild inflammation were observed throughout the CNS, particularly in the brain cortex (Fig. 4A), thalamus (Fig. 4B), cerebellum (Fig. 4C) and lumber spinal cord (Fig. 4D). Furthermore, necrotic or degenerated neurons were also observed in the maxillary plexus (Fig. 4E) and intestinal plexus (Fig. 4F). All degenerated cells examined were TBEV-antigen positive (Fig. 4A–F, insets). Mock-infected mice showed no neuronal degeneration, TBEV antigens or inflammation (Fig. 3G–I).

These results strongly suggest that early death following intracerebral infection primarily results from acute neurological dysfunction throughout the CNS directly due to viral cytopathic effects, as observed in subcutaneous infection with high challenge doses [9].

Systemic levels of corticosterone and TNF-α: We previously showed that low dose subcutaneous infections resulted in increased levels of corticosterone and TNF-α in addition to the development of CNS disease. We therefore investigated the levels of corticosterone and TNF-α in the serum following intracerebral infection. The levels of corticosterone were significantly increased in the TBEV-infected mice at 6 days pi compared with the mock-infected mice (Fig. 5A), indicating that the TBEV-infected mice exhibited a severe stress response. On the other hand, the levels of TNF-α were not increased in the TBEV-infected mice compared with the mock-infected mice (Fig. 5B). Thus, increased TNF-α is not a specific response following intracerebral infection.

DISCUSSION

In this study, we demonstrated that following intracerebral infection all mice showing clinical signs of illness subsequently died and that early death resulted from the acute and widespread neuronal degeneration caused by viral cytopathic effects in the CNS. These results suggest that even low dose challenge rapidly reached a lethal level in the CNS and that CNS pathology is directly linked to the lethality; thus, the mortality rate is dose-dependent.

On the other hand, our previous studies showed that peripheral infection with the Oshima strain of TBEV caused dose-independent mortality [2, 9]. Furthermore, depending on the dose of virus administered, a proportion of the mice died either early or late, or recovered following the onset of CNS disease [9]. Early death followed high dose challenge, and clinically, these infections were acute and occurred throughout the CNS. On the other hand, late death followed low dose challenge, and the development of CNS pathology alone did not determine fatality, suggesting that mortality results from a combination of CNS pathology, systemic stress and inflammatory response [9]. These findings indi-
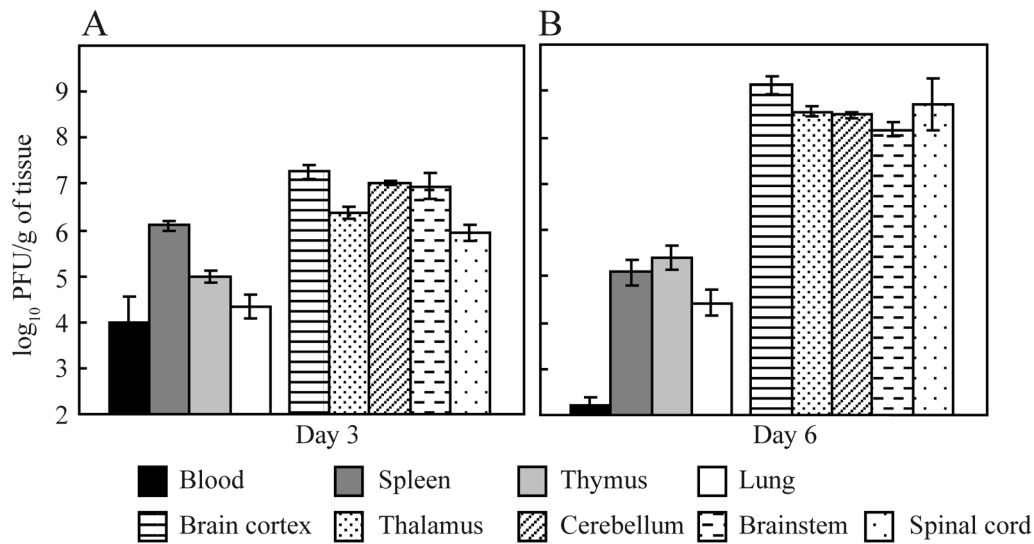
between the challenge doses (Fig. 2B). These results indicate that mice died acutely within 3 to 5 days following the onset of disease. Of note, the morbidity rates were consistent with the mortality rates (Fig. 1B), as all sick mice subsequently died.

Virus replication in mice: Next, we followed the development of viral load in mice after intracerebral infection. At 3 days pi following 10^5 PFU dose challenge, the viral loads were 10^4 to 10^6 PFU/g of tissues in peripheral organs and 10^6 to 10^7 PFU/g of tissues in the CNS (Fig. 3A), suggesting that virus replication occurred almost simultaneously in peripheral and CNS organs. At day 6 pi, the viral loads in the CNS were significantly increased to 10^8 to 10^{10} PFU/g of tissue, whereas those in peripheral organs were unchanged or had decreased (Fig. 3B). There were no significant differences between the viral loads in the brain cortex, thalamus, cerebellum, brain stem and spinal cord (Fig. 3A and 3B).

These data suggest that intracerebral infection induces

Fig. 3.   Virus replication in peripheral organs and the CNS following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on day 3 (A) and day 6 (B). Titers per g of tissue represent the averages from three mice in peripheral organs (blood, lung, thymus and spleen) and the CNS (regions of brain cortex, thalamus, cerebellum, brain stem and spinal cord). Error bars indicate the standard errors (n=3).
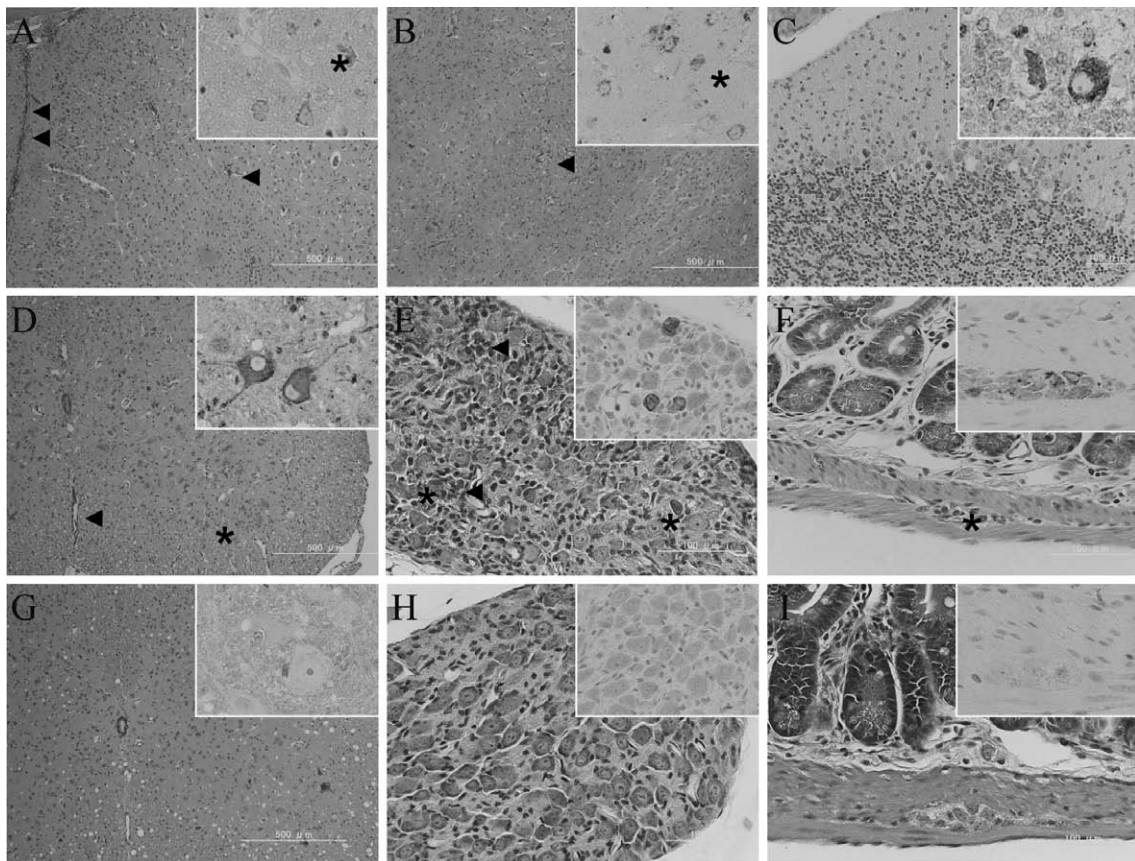

Fig. 4.   Histopathological features in the CNS and neruoplexuses following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on day 8. Inflammation was observed around small blood vessels (cuffing) and the meningis (meningitis) in the brain cortex (A), thalamus (B), lumber spinal cord (D) and maxillary plexus (E; arrow heads). Necrotic or degenerated neurons are indicated (A to F; asterisks). Some Purkinje cells in the cerebellum showed necrosis and neuronal loss (C). Most neurons in the intestinal plexus showed degeneration (F, asterisks). TBEV antigens were detected using E-protein specific TBEV antibody (insets). Each experiment represents three mice.
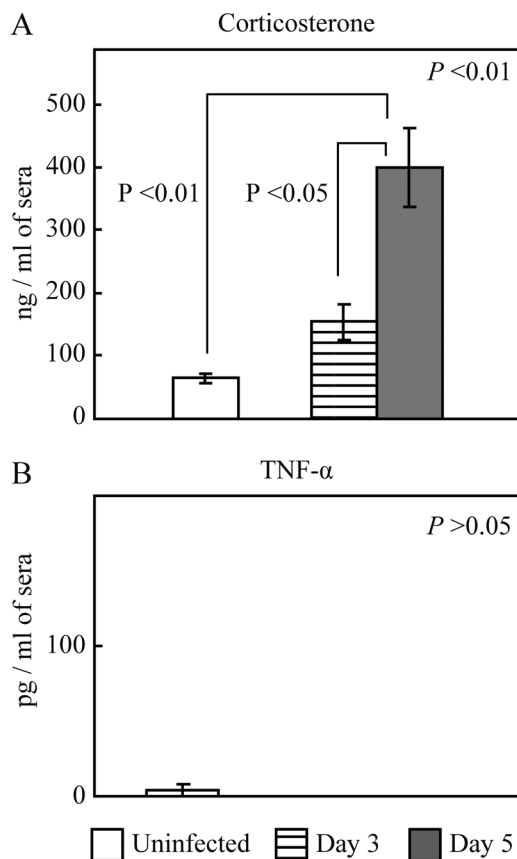
A Corticosterone

B TNF-α

☐ Uninfected ⊟ Day 3 ■ Day 5

Fig. 5. The levels of corticosterone (A) and TNF-α (B) in the sera of mice following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on days 3 and 5. Titers per g of tissue represent averages from three mice. The error bars indicate the standard errors (n=3). The p value in each graph was determined by the analysis of variance, and the significant differences between the pairs were determined by Student's t-test.

cate that the mechanism of fatal infection is fundamentally different between intracerebral and peripheral infection.

Our previous data demonstrated that increased levels of systemic corticosterone and TNF-α contribute to the mechanism of late mortality following subcutaneous infection with low doses [9]. In the present study, the systemic corticosterone levels significantly increased following intracerebral infection (Fig. 5A), indicating that a severe systemic stress response appears to be a common factor in the lethal process of both subcutaneous and intracerebral infections. On the other hand, the levels of TNF-α did not change following intracerebral infection (Fig. 5B). Furthermore, TNF-α also remained unchanged in the early days following subcutaneous infection [9], suggesting that TNF-α increases only later in the disease progression.

In human cases, death usually occurs within 5 to 7 days of the onset of neurological symptoms [4]. It is usually believed that human cases succumb to acute and critical

neuronal dysfunction following direct viral infection of the neurons. In the present study, we showed that neurons were specific target cells of TBEV infection in the CNS and that the mice likely died due to direct viral cytopathic effects throughout the CNS. Thus, early death possibly relates to the mechanism of mortality in human cases. In addition, although adequate information on the systemic stress and inflammatory response correlated with fatal cases in humans is lacking, our results indicate that systemic responses in the late phase potentially contribute to the severity and fatality of TBE in human cases. Further elucidation of the mechanism of death in the mouse model is an important priority in development of effective treatment strategies for human TBE.

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