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A Murine Model of Dengue Virus-induced Acute Viral Encephalitis-like Disease

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Nov. 14, 2018

Dr. Vineeta Bajaj, Review Editor
Journal of Visualized Experiments

Dear Professor Bajaj,
Enclosed is our revised manuscript JoVE59132R1 titled: "A Murine Model of Dengue Virus-induced Acute Viral Encephalitis-like Disease". We deeply look forward to seeing this manuscript is suitable for consideration for publication in the *Journal of Visualized Experiments*. We made revision in the re-submitted manuscript and we are grateful for the opportunity to revise our works and thank the reviewers and editors for the time and effort they took to critique our work and offer suggestions for improving it. Our point-by-point responses to the referees' comments are also enclosed. We hope that you now find our manuscript suitable for publication.

We look forward to your reply.
Yours respectfully,

Chiou-Feng Lin, Ph.D.
Corresponding Author

TITLE:

A Murine Model of Dengue Virus-induced Acute Viral Encephalitis-like Disease

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KEYWORDS:

Murine, dengue virus, encephalitis, neurotoxicity, intracerebral injection, intraperitoneal injection, suckling mice

SUMMARY:

Here, we present a protocol for creating an immunocompetent ICR (Institute of Cancer Research) murine model of central nervous system infection to display the development of neuropathy. Monitoring acute viral encephalitic disorders by identical disease scores could be performed for showing dengue-virus-induced neuropathy in vivo.

ABSTRACT:

Dengue virus (DENV), an arthropod-borne virus transmitted by mosquitoes, may cause the severe disease known as dengue hemorrhagic fever, which is characterized by lethal complications due to plasma leakage, ascites, pleural effusion, respiratory distress, severe bleeding, and organ impairment. A few cases of DENV infection present neurological manifestations; however, studies have not explored DENV-induced neuropathogenesis further. In this study, we present a protocol to use an immunocompetent outbred ICR (Institute of Cancer Research) mouse for

investigating the induction of central nervous system (CNS) infection with DENV, followed by the progression of acute viral encephalitis-like disease.

INTRODUCTION:

DENV, an arthropod-borne virus of the *Flaviviridae* family, contains a positive-sense RNA genome that encodes three viral structural proteins (capsid, premembrane, and envelope) and seven viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The four serotypes of DENV (DENV1–4), which infect approximately 390 million people annually, cause a global burden even though governments have directed substantial efforts toward mosquito vector and disease control[1]. Currently, protective vaccines and therapeutic antiviral drugs are under development and require further long-term validation[2]. In clinical practice, although a dengue patient with CNS infection is rare, it needs to be further explored to understand the diversity of dengue disease development[3]. Further investigation and validation are needed; notably, the World Health Organization (WHO) has included the involvement of CNS impairment, such as cognitive impairment, convulsions, encephalopathy, and encephalitis, in the classification of severe dengue[3,4]. Constructing animal models of DENV infection is indispensable for exploring the neuropathogenesis of DENV infection.

For generating CNS infection by DENV, several studies have executed different routes of DENV infection, including (1) intracerebral inoculation of C57BL/6 mice who received 4 x 10^3 plaque-forming units (PFU) of nonadapted DENV3[5,6], (2) intraperitoneal inoculation of BALB/c mice who received 7 x 10^4 PFU of in vitro neuroadapted DENV4[7], (3) intracerebral inoculation of Swiss mice who received 1 x 10^5 PFU of in vivo neuroadapted DENV1[8], and (4) intracerebral and intraperitoneal co-inoculation of ICR suckling mice who received 1 x 10^6 PFU of nonadapted DENV2[9]. According to the findings of these studies[5–9], DENV infection in mice result in viral replication in the brain, leading to acute viral encephalitis-like syndromes, behavioral changes accompanied by limb paralysis and postural instability, CNS neurotoxicity and inflammation, general and localized plasma leakage through the blood-brain barrier (BBB), and lethality. All the results from these studies[5–9] have shown the ability of DENV to infect the CNS and the induction of acute viral encephalitis-like disease following infection.

Based on our current findings[9–15], we have created a murine model of DENV infection as an in vivo platform to examine the therapeutic efficacy of targeted agents/factors against viral replication, as well as neurotoxicity. Here, we report the protocol utilized to create an immunocompetent outbred ICR mouse to study CNS infection and to monitor the development of neuropathies with different severities caused by DENV. The results show the significant progression of encephalitis-like disease in DENV-infected mice in a time-dependent manner.

PROTOCOL:

Experimental protocols of animal study were approved by the Institutional Animal Care and User Committee of the National Defense Medical Center (IACUC number: 16-261), according to guidelines established by the Ministry of Science and Technology, Taiwan.

1. Infection procedure

1.1. Prepare nonadapted DENV2 (strain PL046) stocks[9] (originally obtained from the Centers for Disease Control in Taiwan, ranging from 2.5 x 10^7 to 1 x 10^9 PFU/mL).

1.2. Dilute the virus stock to 1 x 10^6 PFU with Roswell Park Memorial Institute (RPMI) 1640 medium to a total volume of 40 µL.

1.3. Fill one 0.3 mL syringe fitted with a 30 G needle with 10 µL (2.5 x 10^5 PFU) of diluted virus, and fill another 0.3 mL syringe fitted with a 30 G needle with 30 µL (7.5 x 10^5 PFU) of diluted virus.

1.4. Hold the 7-day-old ICR suckling mouse and conduct the following steps.

1.4.1. For the intracerebral injection, hold the mouse in a prone position by pressing the auricle between the index finger and thumb, and intracerebrally inject 10 µL of diluted virus into the lambda area, the point at the intersection of the sagittal and lambdoid suture[16].

1.4.2. After the intracerebral injection, hold the mouse in a supine position by using the index finger and thumb and intraperitoneally inject 30 µL of diluted virus gently into the murine abdomenl.

NOTE: To avoid cannibalism, 75% alcohol is used to create a temporary loss of the mother's olfactory sensation. Additionally, covering the litters with their mother's stool and urine is suggested.

1.5. Lay the suckling mice back in their cages and wait for 5 min to check their safety poststimulation with the avidity of mice, including walking and mother milk sucking.

NOTE: Most often, mice demonstrate normal activity poststimulation and there does not seem to be any harmful effects following the technical challenge. During this experiment, the animals were maintained by the Institutional Animal Care and User Committee of the National Defense Medical Center, Taiwan.

1.6. Evaluate the daily progress of the mice in terms of body weight (by using a microgram balance), acute viral encephalitis-like disease (by disease scoring as described in section 2), and survival rate[9–15].

2. Disease scoring

2.1. Monitor the grade of acute viral encephalitis-like illnesses. Assign a score of 0 to healthy mice; 1 to mice with minor illness symptoms, including weight loss, reduced mobility, and a hunchback body orientation; 2 to mice that exhibit limbic seizure; 3 to mice the exhibit limbic weakness, including moving with difficulty and anterior limb or posterior limb weakness; 4 for paralysis; 5 for death.

NOTE: Once the score reaches 3, the disease symptoms are considered to be evolving rapidly.

2.2. Plot the scoring of the disease, according to step 2.1, for each day as a curve-based figure by using the mean ± SD of the daily test scores in each group.

REPRESENTATIVE RESULTS:

Severe dengue-associated neurological complications have been reported in patients for dengue pathogenesis[4]. Although these cases are rare in the clinic, creating an immunocompetent murine model of DENV infection can be used not only for studying immunopathogenesis but also for exploring CNS infection, neuroinflammation, neurotoxicity, and acute viral encephalitis-like disease. In this study, according to our current model[9–15], 7-day-old ICR suckling mice were inoculated concurrently with DENV2 through an intracerebral microinjection (2.5×10^5 PFU) and an intraperitoneal injection (7.5×10^5 PFU) (**Figure 1**). Following infection, we performed numerous analyses to show the presence of dsRNA replication, viral protein expression, and virus release in the DENV-infected brain[9–15]. These experiments demonstrated that a successful in vivo model of DENV infection, replication, and release has been established.

To evaluate the induction of acute viral encephalitis-like illnesses[9–15], we monitored the changes in body weight in DENV-infected mice. Compared with the body weight changes in the mock group (where the mice were inoculated with RPMI medium only), **Figure 2** shows an increase in the mock group but a significant ($P < 0.01$) decrease in body weight in the infected mice. According to the symptoms of encephalitis-like illnesses, including hunchback posture, limbic seizure, limbic weakness, paralysis, and death, as manifested in DENV-infected mice, **Figure 3** shows a significant ($P < 0.05$) increase in clinical score in the DENV-infected mice. The survival assay in **Figure 4** shows a time-dependent reduction in the survival rate ($P < 0.001$) of the DENV-infected mice. The data indicate the establishment of an infectious model of DENV in the CNS, which shows the progression of acute viral encephalitis-like illnesses.

FIGURE LEGENDS:

Figure 1: Experimental design for dengue virus (DENV) infection in suckling mice. Seven-day-old ICR suckling mice were inoculated with DENV2 PL046 by concurrent intracranial and intraperitoneal injections. We measured changes in body weight, disease score, and mortality.

Figure 2: The body weight in suckling mice during dengue virus (DENV) infection. In DENV2-infected ICR suckling mice ($n = 12$), changes in body weight were measured per day. Following a Wilcoxon signed-rank test, the values are presented as the mean ± SD. $^{**}p < 0.01$.

Figure 3: The encephalitic disease score in suckling mice during dengue virus (DENV) infection. Following DENV2 infection in ICR suckling mice ($n = 12$), time-kinetic changes in clinical scores for hunchback posture (score 1), limbic seizure (score 2), limbic weakness (score 3), paralysis (score 4), and death (score 5) were measured. Following a Wilcoxon signed-rank test, the values are presented as the mean ± SD. $^{*}p < 0.05$.

Figure 4: The survival rate in suckling mice during dengue virus (DENV) infection. Time-kinetic changes in survival rates were measured in DENV2-infected ICR suckling mice (*n* = 12). Following a log-rank test, the values are presented as the mean ± SD. ***$p < 0.001$.

DISCUSSION:

DENV infection has been detected in the CNS of severe dengue patients[3,17], suggesting the possibility of the manifestation of acute viral encephalitis occurred during dengue pathogenesis. Here, we report an in vivo murine model of DENV infection for studying the involvement of CNS dysfunction in severe dengue, particularly with a focus on DENV-induced acute viral encephalitis-like illnesses. As compared with the previous models, particularly for one-route infection (intravenous only, intraperitoneal only, or intracerebral only), an extremely high titer (1×10^8 PFU) of DENV is utilized in immunocompetent mice. However, in immunocompromised mice, a relatively low but variant titer (1×10^5 to 1×10^8 PFU) of DENV can be performed[18–20]. In this study, we used two-route infection to induce concurrent CNS and systemic infection with a low titer (intracranial: 2.5×10^5 PFU; intraperitoneal: 7.5×10^5 PFU) of DENV in immunocompetent ICR suckling mice. The progression of encephalitis-like disease is completely reproducible[9–15]. The possible limitation of this study is, therefore, mitigated by using a concurrent intracranial and intraperitoneal injection. Although it is artificial—however, consistent with previous works by others[5–8] and us[9–15]—DENV is actively replicated in the experimental murine brain[9–15], and the possible effects of viral factors and host responses on neuroinflammation, as well as neurotoxicity, are, therefore, of interest.

For neuropathogenesis, the stimulation of neuronal cell apoptosis is triggered by DENV infection as well as CNS inflammation, such as microglial cell activation followed by proinflammatory cytokine tumor necrosis factor-α (TNF-α) production[8,12,15]. Direct neurotoxicity caused by DENV infection and indirect neuroinflammation initiated by host immune responses are speculated to be involved in dengue encephalitis. Acute viral encephalitis is caused by virus-induced CNS inflammation of the brain[3,21]. Common clinical symptoms are a high fever, headache, stiff neck and back, vomiting, and confusion, while severe cases also develop seizures, paralysis, and coma[21]. By monitoring the changes in scores with hunchback posture, limbic seizure, limbic weakness, paralysis, and death, the development of these illnesses shows the progression of acute viral encephalitis-like disease in DENV-infected mice[9–15]. Generally, those DENV-infected mice with a score of 3 (according to the disease scoring method presented in the protocol) rapidly progressed to paralysis and death. The pathogenic mechanisms causing these illnesses require further investigation; however, it is speculated that neuronal dysfunction followed by neuropathy induction is involved[21]. Targeting viral replication, as well as TNF-α-mediated CNS inflammation[10–14], at least in part, confers therapeutic effects against the progression of dengue-associated acute viral encephalitis-like disease. The protocol of this study could be used for creating an encephalitis-like pathological model of DENV infection.

Some studies have described the induction of intracerebral hemorrhage induced by DENV infection in the brain[22,23]. We previously showed the induction of BBB disruption in DENV-infected mice[9]. Although DENV is not recognized as a neurotropic virus, DENV can produce an effective infection in neuronal cells in vivo and in vitro[8,11]. Dopamine D2 receptor (D2R) is

speculated to be a neuronal receptor for DENV infection, and pharmacologically targeting D2R effectively reduces DENV infection in neuronal cells in vivo and in vitro, as well as DENV-induced encephalitic illnesses[11,24]. Possible therapeutic strategies against acute viral encephalitis are important to develop so that severe dengue cases with neurological complications can be treated. For its future application, this protocol of a disease model using DENV infection in the brain may become an in vivo platform to screen not only the viral and host factors associated with neuropathogenesis but also possible antiviral and anti-encephalitic drugs. Regarding the incidence of variant neurological disorders caused by DENV infection[25] by this established protocol, the difference between the variable types and/or strains of DENV for causing neurological dysfunction is needed for validation.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

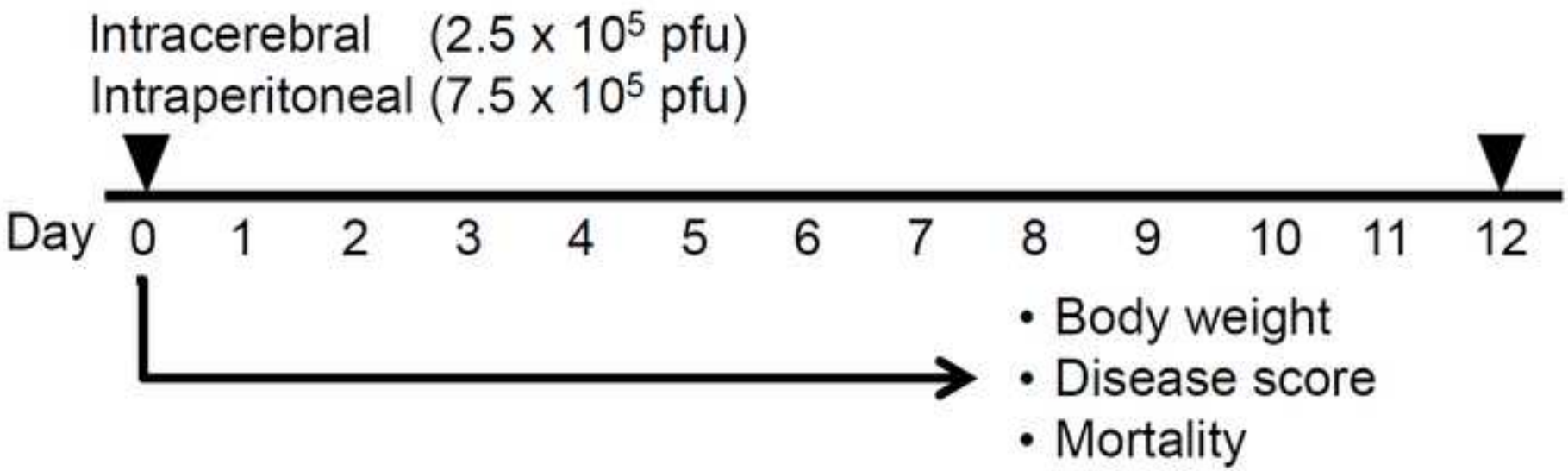


Figure 2

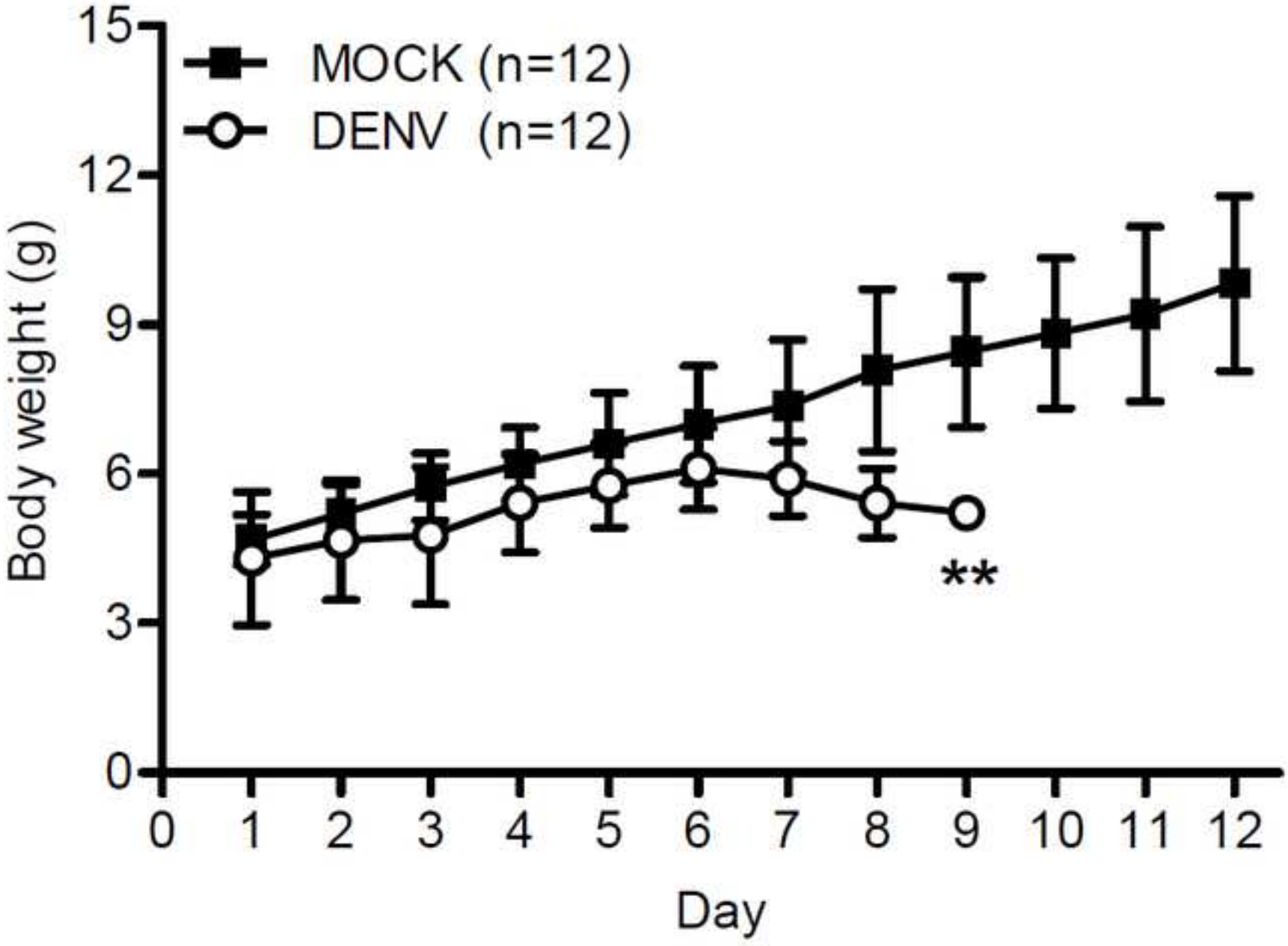


Figure 3

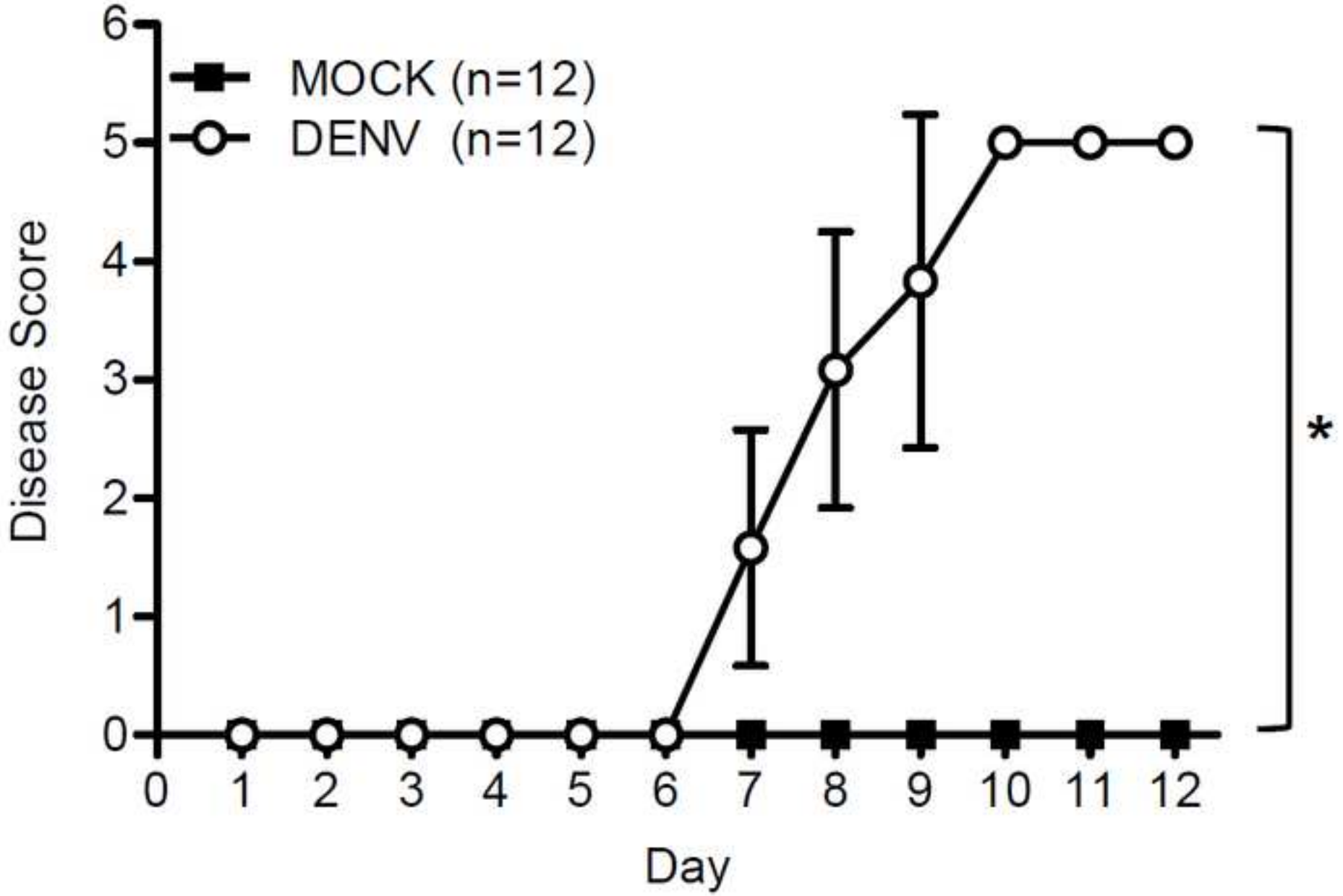
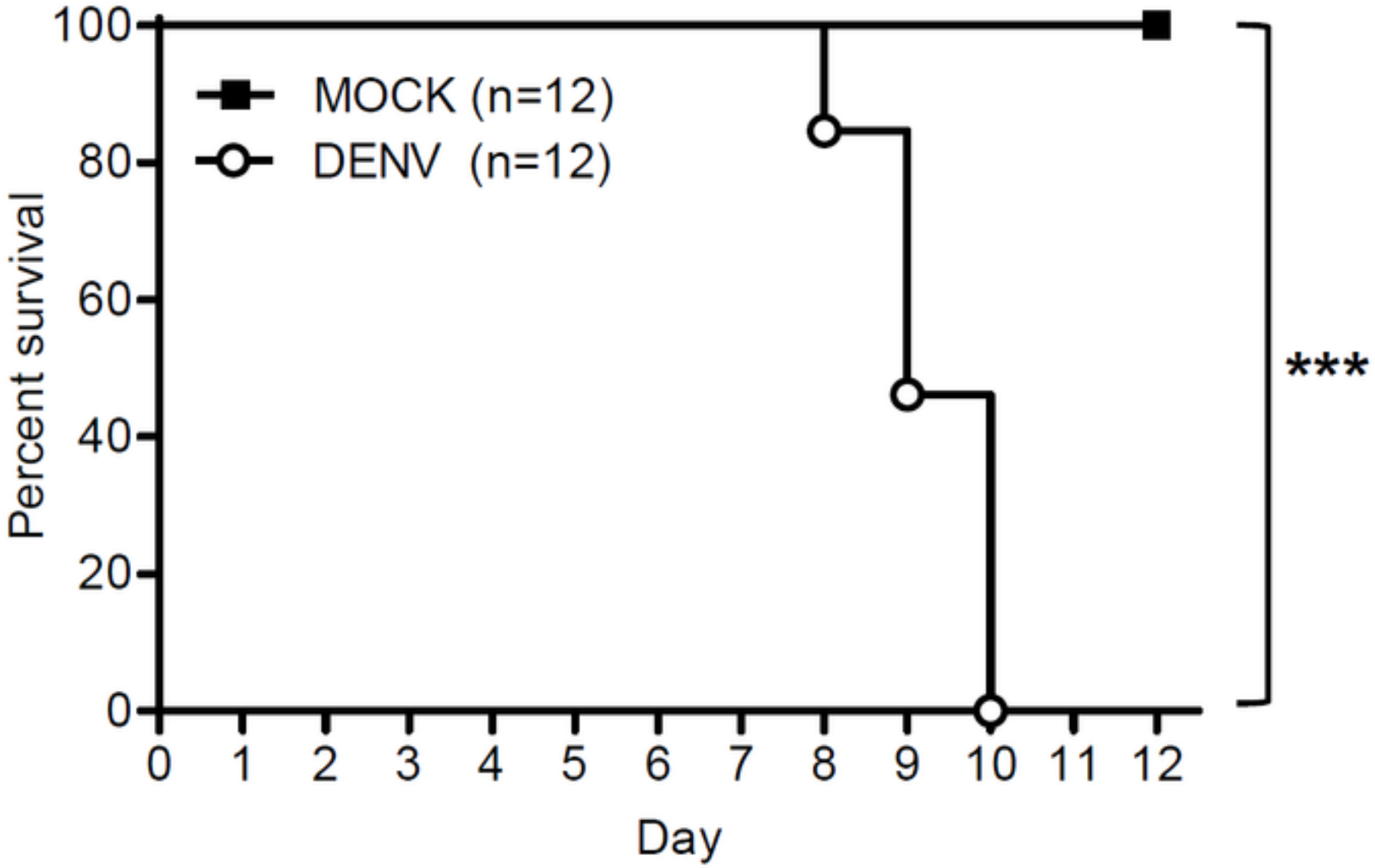


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Roswell Park Memorial Institute 1640 medium (RPMI)	Gibco	11875-085	Diluting virus
0.3-mL Insulin Syringe	BD Ultra-Fine-II	328838	Intracerebral and intraperitoneal injection
Microbalance	METTLER TOLEDO's LabX	AL104	Weight mouse
Non-adapted DENV2 (strain PL046)	The Centers for Disease Control of Taiwan	-	Infect mouse
Institute of Cancer Research (ICR) suckling mouse	BioLASCO Taiwan Co., Ltd	-	Our murine model

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Editorial comments:

A1: TITLE PAGE (line 2) "The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please have a proficient English speaker thoroughly review the manuscript and edit any errors. I have marked the sentences that are hard to understand in green color."

REPLY: The sentences with grammatical errors have been edited as you mentioned.

A2: SUMMARY (line 46-49) Needs revisions for language. As it is written, it is hard to understand what is being said.

REPLY: The sentences have been edited as "Here, we prepare a protocol for creating an immunocompetent ICR murine model of CNS infection to display the development of neuropathy. Monitoring acute viral encephalitic disorders by the identical disease scores could be performed for showing DENV-induced neuropathy in vivo." (Line 46-49).

A3: ABSTRACT (line 59-60) Do you mean detecting viral replication?

REPLY: The sentence has been deleted. This study focused on the creation of infectious model and disease scoring.

A4: ABSTRACT (line 60-62) Needs revisions for language.

REPLY: The sentence has been deleted.

A5: INTRODUCTION (line 84-87) Needs references.

REPLY: The sentences have been edited as "According to the findings [5-9], DENV infection in mice ……." (Line 81).

A6: INTRODUCTION (line 87) References?

REPLY: The sentences have been edited as "All of results from these studies [5-9] ……." (Line 84-85).

A7: PROTOCOL 1.1 (line 104) Please add it of the table of materials.

REPLY: The materials have been added in the Table of Materials.

A8: PROTOCOL 1.2 (line 107) Add to the table of materials.

REPLY: The materials have been added in the Table of Materials.

A9: PROTOCOL 1.4 (line 114) Add mouse strain to the table of materials.

REPLY: The materials have been added in the Table of Materials.

A10: PROTOCOL 1.5 (line 118-120) Is this the same mouse? It will be better if you organize step 1.4 and 1.5 in a logical manner, instead of the parallel description of 2 types of injections.
REPLY: The sentences have been corrected as you mentioned (Line 111-118).

A11: PROTOCOL 1.7 (line 131) Please expand and describe the measurements. How are the animals maintained during the testing phase?
A12: PROTOCOL 1.7 (line 131-132) How was this checked for? Please describe the signs.
REPLY: The information has been added as you mentioned (Line 129-132) as "Animals are maintained in the Institutional Animal Care and User Committee of National Defense Medical Center, Taiwan. We next evaluate the daily progress of the mice in terms of body weight (by using Microbalance), acute viral encephalitis-like disease (by disease scoring as described below), and survival rate accordingly.".

A13: REPRESENTATIVE RESULTS (line 149) In humans?
REPLY: The sentences have been corrected as you mentioned (Line 147-148).

A14: REPRESENTATIVE RESULTS (line 162) Define this group, what was injected?
REPLY: The group has been corrected as you mentioned (Line 161-162).

A15: FIGURE LEGENDS (line 171) Please remove the text "Shen et al. Figure #" from all figures.
REPLY: The figures have been corrected as you mentioned.

A16: DISCUSSION (line 192) Do you mean detected?
REPLY: The sentence has been corrected as you mentioned (Line 192).

A17: DISCUSSION (line 198-199) References?
REPLY: The references have been added (Line 199).

A18: DISCUSSION (line 205) This was not demonstrated in the current manuscript. Please cite your previously published work where you reported this.
REPLY: The references have been added (Line 205).

A19: DISCUSSION (line 212-213) References?
REPLY: The references have been added (Line 213).

A20: DISCUSSION (line 224) I am not sure what is being said, it however appears unsupported by this article.

REPLY: The sentence has been corrected (Line 224).

TITLE:

A Murine Model of Dengue Virus-induced Acute Viral Encephalitis-like Disease

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KEYWORDS:

Murine, dengue virus, encephalitis, neurotoxicity, intracerebral injection, intraperitoneal injection, suckling mice

ABBREVIATIONS:

BBB	blood-brain barrier
CNS	central nervous system
D2R	dopamine D2 receptor
DENV	dengue virus
ICR	Institute of Cancer Research
NS	nonstructural protein
PFU	plaque forming units
RNA	ribonucleic acid
SD	standard deviation
TNF-α	tumor necrosis factor α
WHO	World Health Organization

SUMMARY:

Here, we prepare a protocol for creating an immunocompetent ICR murine model of CNS infection to display the development of neuropathy. Monitoring acute viral encephalitic disorders by the identical disease scores could be performed for showing DENV-induced neuropathy in vivo.

ABSTRACT:

Dengue virus (DENV), an arthropod-borne virus transmitted by mosquitoes, may cause the severe disease known as dengue hemorrhagic fever, which is characterized by lethal complications due to plasma leakage, ascites, pleural effusion, respiratory distress, severe bleeding, and organ impairment. A few cases of DENV infection present with neurological manifestations; however, studies have not explored DENV-induced neuropathogenesis further. In this study, we present a protocol to use an immunocompetent outbred Institute of Cancer Research (ICR) mouse for investigating the induction of central nervous system (CNS) infection with DENV followed by the progression of acute viral encephalitis-like disease.

INTRODUCTION:

Dengue virus (DENV), an arthropod-borne virus of the *Flaviviridae* family, contains a positive-sense RNA genome that encodes three viral structural proteins (capsid, premembrane, and envelope) and seven viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The four serotypes of DENV (DENV1-4), which infect approximately 390 million people annually, cause a global burden even though governments have directed substantial efforts toward mosquito vector and disease control [1]. Currently, protective vaccines and therapeutic antiviral drugs are under development and require further long-term validation [2]. In clinical practice, although dengue patient with central nervous system (CNS) infection is rare, it is needed for further exploring the diversity of dengue disease development [3]. Further investigation and validation are needed; notably, the World Health Organization (WHO) has included the involvement of CNS impairment, such as cognitive impairment, convulsions, encephalopathy, and encephalitis, in the classification of severe dengue [3,4]. Constructing animal models of DENV infection is indispensable for exploring the neuropathogenesis of DENV infection.

For generating CNS infection by DENV, several studies have executed different routes of DENV infection, including (1) intracerebral inoculation of C57BL/6 mice who received 4×10^3 plaque forming units (PFU) of non-adapted DENV3 [5,6], (2) intraperitoneal inoculation of BALB/c mice who received 7×10^4 PFU of in vitro neuroadapted DENV4 [7], (3) intracerebral inoculation of Swiss mice who received 1×10^5 PFU of in vivo neuroadapted DENV1 [8], and (4) intracerebral and intraperitoneal coinoculation of ICR suckling mice who received 1×10^6 PFU of non-adapted DENV2 [9]. According to the findings [5-9], DENV infection in mice result in viral replication in the brain, leading to acute viral encephalitis-like syndromes, behavioral changes accompanied by limb paralysis and postural instability, CNS neurotoxicity and inflammation, general and localized plasma leakage through the blood-brain barrier (BBB), and lethality. All of results from these studies [5-9] have shown the ability of DENV to infect the CNS and the induction of acute viral encephalitis-like disease following infection.

Based on our current findings [9-15], we have created a murine model of DENV infection as an *in vivo* platform to examine the therapeutic efficiency of targeted agents/factors against viral

replication as well as neurotoxicity. Here, we report the protocol we utilized to create an immunocompetent outbred ICR mouse to study CNS infection and to monitor the development of neuropathies with different severities caused by DENV. The results show the significant progression of encephalitis-like disease in DENV-infected mice in a time-dependent manner.

PROTOCOL:

Experimental protocols of animal study according to guidelines established by the Ministry of Science and Technology, Taiwan were approved by the Institutional Animal Care and User Committee of National Defense Medical Center (IACUC number: 16-261).

1. Infection Procedure

1.1. Prepare non-adapted DENV2 (strain PL046) stocks [9], originally obtained from the Centers for Disease Control in Taiwan, ranging from 2.5×10^7 to 1×10^9 PFU/ml.

1.2. Dilute the virus stock to 1×10^6 PFU with Roswell Park Memorial Institute 1640 medium (RPMI) to a total volume of 40 µL.

1.3. Fill one 0.3-mL syringe fitted with a 30-gauge needle with 10 µL (2.5×10^5 PFU) of diluted virus, and fill another 0.3-mL syringe fitted with a 30-gauge needle with 30 µL (7.5×10^5 PFU) of diluted virus.

1.4. Seven-day-old ICR suckling mouse was held and conducted the following steps:

1.4.1. For intracerebral injection, mice was held in a prone position by pressing the auricle between the index finger and thumb, and intracerebrally injected 10 µL diluted virus into the lambda area, the point at the intersection of the sagittal and lambdoid suture [16].

1.4.2. After intracerebral injection, mice was held in a supine position by using the index finger and thumb and intraperitoneally injected 30 µL diluted virus into the murine abdominal gently.

NOTE: To avoid cannibalism, 75% alcohol is used to make temporarily loss on mother's olfactory sensation. Additionally, covering the litters with their mother's stools and urines is suggested.

1.5. Lay the suckling mice back in their cages and wait for 5 min to check their safety poststimulation with the avidity of mice, including walking and mother milk sucking. Basically, mice represent normal activity poststimulation without harmful effects following the technical challenge.

1.6. Animals are maintained in the Institutional Animal Care and User Committee of National Defense Medical Center, Taiwan. We next evaluate the daily progress of the mice in terms of body weight (by using Microbalance), acute viral encephalitis-like disease (by disease scoring as described below), and survival rate accordingly [9-15].

2. Disease Scoring

2.1. Monitor the grade of acute viral encephalitis-like illnesses as Score 0 for healthy mice; Score 1 for minor illness including weight loss, reduced mobility, and a hunchback body orientation; 2 for limbic seizure; Score 3 for limbic weakness, including moving with difficulty and anterior limb or posterior limb weakness; Score 4 for paralysis; and Score 5 for death.

NOTE: Once the score reaches 3, the disease symptoms are considered to be evolving rapidly.

2.2. Plot disease scoring, according to 2.2., for each day as a curve-based figure by using the mean ± SD of the daily test scores in each group.

REPRESENTATIVE RESULTS:

Severe dengue-associated neurological complications have been reported in patients for dengue pathogenesis [4]. Although the cases are rare in clinic, creating an immunocompetent murine model of DENV infection can be used not only for studying immunopathogenesis but also for exploring CNS infection, neuroinflammation, neurotoxicity, and acute viral encephalitis-like disease. In this study, according to our current model, as summarized in the Protocol-Infectious Procedure section [9-15], seven-day-old ICR suckling mice were inoculated concurrently with DENV2 through intracerebral microinjection (2.5×10^5 PFU) and intraperitoneal injection (7.5×10^5 PFU) (**Figure 1**). Following infection, we performed numerous analyses to show the presence of dsRNA replication, viral protein expression, and virus release in DENV-infected brain [9-15]. These experiments demonstrated that we established a successful *in vivo* model of DENV infection, replication, and release.

To evaluate the induction of acute viral encephalitis-like illnesses, as shown in the Protocol-Disease Scoring section [9-15], we monitored the changes in body weight in DENV-infected mice. Compared with the body weight changes in the mock group (mice were inoculated with RPMI medium only), **Figure 2** shows an increase in mock group but a significant ($P < 0.01$) decrease in body weight in infected mice. According to the symptoms of encephalitis-like illnesses, including hunchback posture, limbic seizure, limbic weakness, paralysis, and death, as existed in DENV-infected mice, **Figure 3** shows a significant ($P < 0.05$) increase in clinical score in the DENV-infected mice. The survival assay in **Figure 4** shows a time-dependent reduction in the survival rate ($P < 0.001$) of the DENV-infected mice. The data indicate the establishment of an infectious model of DENV in the CNS, which shows the progression of acute viral encephalitis-like illnesses.

FIGURE LEGENDS:

Figure 1. Experimental design for dengue virus (DENV) infection in suckling mice. Seven-day-old ICR suckling mice were inoculated with DENV2 PL046 by concurrent intracranial and intraperitoneal injections. We measured changes in body weight, disease score, and mortality.

Figure 2. The body weight in suckling mice during dengue virus (DENV) infection. In DENV2-infected ICR suckling mice (*n*=12), changes of body weight per day were measured. Following a Wilcoxon signed rank test, values are presented as the mean ± SD. ** $p < 0.01$.

Figure 3. The encephalitic disease score in suckling mice during dengue virus (DENV) infection. Following DENV2 infection in ICR suckling mice (*n*=12), time-kinetic changes in clinical scores with hunchback posture (score 1), limbic seizure (score 2), limbic weakness (score 3), paralysis (score 4), and death (score 5), were measured. Following a Wilcoxon signed rank test, values are presented as the mean ± SD. * $p < 0.05$.

Figure 4. The survival rate in suckling mice during dengue virus (DENV) infection. Time-kinetic changes in survival rates were measured in DENV2-infected ICR suckling mice (*n*=12). Following a log-rank test, values are presented as the mean ± SD. *** $p < 0.001$.

DISCUSSION:

DENV infection has been detected in the CNS of severe dengue patients [3,17], suggesting the possibility of the manifestation of acute viral encephalitis occurred during dengue pathogenesis. Here, we report an *in vivo* murine model of DENV infection for studying the involvement of CNS dysfunction in severe dengue, particularly with a focus on DENV-induced acute viral encephalitis-like illnesses. As compared with the previous models, particularly for one-route infection (intravenous only, intraperitoneal only, or intracerebral only), an extremely high titer (1 x 10^8 PFU) of DENV is utilized in immunocompetent mice. However, in immunocompromised mice, a relative low but variant titer (1 x 10^5 to 1 x 10^8 PFU) of DENV can be performed [18-20]. In this study, we used two-route infection to induce concurrent CNS and systemic infection with low titer (IC 2.5 x 10^5 PFU and IP 7.5 x 10^5 PFU) of DENV in immunocompetent ICR suckling mice. The progression of encephalitis-like disease is completely reproducible [9-15]. The possible limitation of this study is therefore considered by using a concurrent intracranial and intraperitoneal injection. Although it is artificial, however, consistent with previous works by others [5-8] and ourselves [9-15], DENV is actively replicated in the experimental murine brain [9-15], and the possible effects of viral factors and host responses on neuroinflammation as well as neurotoxicity are therefore of interest.

For neuropathogenesis, the stimulation of neuronal cell apoptosis is triggered by DENV infection as well as CNS inflammation, such as microglial cell activation followed by pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) production [8,12,15]. Direct neurotoxicity caused by DENV infection and indirect neuroinflammation initiated by host immune responses are speculated to be involved in dengue encephalitis. Acute viral encephalitis is caused by virus-induced CNS inflammation of the brain[3,21]. Common clinical symptoms are high fever, headache, stiff neck and back, vomiting, and confusion, while severe cases also develop seizures, paralysis, and coma [21]. By monitoring the changes in scores with hunchback posture, limbic seizure, limbic weakness, paralysis, and death, the development of these illnesses shows the progression of acute viral encephalitis-like disease in DENV-infected mice [9-15]. Generally, as shown in the Protocol-Disease Scoring section, those DENV-infected mice with a score of 3 rapidly progressed to paralysis and death. The pathogenic mechanisms causing these illnesses require further investigation; however, it is speculated that neuronal dysfunction followed by

neuropathy induction is involved [21]. Targeting viral replication as well as TNF-α-mediated CNS inflammation [10-14], at least in part, confers therapeutic effects against the progression of dengue-associated acute viral encephalitis-like disease. The protocol of this study could confer significant application for creating an encephalitis-like pathological model of DENV infection. Some studies have described the induction of intracerebral hemorrhage induced by DENV infection in the brain [22,23]. We previously showed the induction of BBB disruption in DENV-infected mice [9]. Although DENV is not recognized as a neurotropic virus, DENV can produce effective infection in neuronal cells *in vivo* and *in vitro* [8,11]. Dopamine D2 receptor (D2R) is speculated to be a neuronal receptor for DENV infection, and pharmacologically targeting D2R effectively reduces DENV infection in neuronal cells *in vivo* and *in vitro* as well as DENV-induced encephalitic illnesses [11,24]. Possible therapeutic strategies against acute viral encephalitis are important to develop so that severe dengue cases with neurological complications can be treated. For the future application, our protocol of disease model using DENV infection in the brain may become an *in vivo* platform to screen not only the viral and host factors associated with neuropathogenesis but also possible antiviral and anti-encephalitic drugs. Regarding the incidence of variant neurological disorders caused by DENV infection [25], by our established protocol, the difference between the variable types and/or strains of DENV for causing neurological dysfunction is needed to validate.

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DISCLOSURES:

The authors have nothing to disclose.

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