Dengue Virus Infection in Mice

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Abstract : Dengue virus type 2 infection in mice was studied and the results were summarized as follows : (1) The suckling mice younger than 3 days of age were much more susceptible to dengue virus type 2 than the mice older than 5 days of age. The differences of the susceptibility between these mice were demonstrated in the following points. (a) The mice younger than 3 days of age were lethally infected even by the extraneural peripheral routes as well as by the intracerebral route, whereas the mice older than 5 days of age were lethally infected only by the intracerebral route. (b) The mice younger than 3 days of age developed viremia after infection both by intracerebral and intramuscular routes, but the older mice did not. (c) The infection of the mice younger than 3 days of age by intracerebral route gave rise to the virus replication in all organs of 9 species tested but in the older mice, virus replication occurred only in the brain and bone marrows. (2) Interferon-like substances were induced only in the brain after infection in every age group of mice, but this induction appeared to play no role in the outcome of infection of mice, reflecting simply the rate of virus replication in the organ. (3) Virus transmission from viremic suckling mice to the adult female *Ae. aegypti* and from the infected mosquitoes to suckling mice was demonstrated, although the latter transmission resulted in inapparent infection of the mice.

INTRODUCTION

It is a well known fact that dengue hemorrhagic fever and dengue shock syndrome are followed by the infection of dengue virus. However, the pathogenesis of these sickness still remains unresolved although various explanations for these deviant syndromes have been advanced, which are summarized by Halstead (1970).

One of the approarch to the problem would be the development of test measures or animal model by which the pathogenesis of dengue and the pathogenicity of virus strains can be studied.

Although monkeys and chimpanzees are used in recent works of dengue viruses (Rosen, 1958 : Halstead *et al*, 1973 : Tarr *et al*, 1976 : Scherer *et al*, 1978), numerous studies of dengue virus using small laboratory animals concerning the establishment of animal model and the pathogenicity of the virus have been reported (Sabin, 1952 : Sabin and Schlesinger, 1945 : Hotta, 1952 : Meiklejohn *et al*, 1952 : Meiklejohn *et al*, 1952b : Schlesinger and Frankel, 1952 : Cole and Wisseman, 1969 : Chaturvedi *et al*, 1978). The results of those studies can be summarized as follows : 1. The mouse is the only susceptible small experimental animal for

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dengue virus with the exception of suckling hamster (Meiklejohn *et al*, 1952). 2. Suckling animals are more susceptible to the virus than adults. 3. Intracerebral introduction of the virus is the only method to cause sickness with a few exceptions such as the intranasal infection in ground squirrel (Hiroki *et al*, 1944) and the intraperitoneal infection in albino rats (Tsurumi *et al*, 1943). 4. The pathogenicity of the virus to mice increases with the repeated passages of the virus in the mouse brain.

The present study was undertaken to obtain the additional basic data concerning the susceptibility of mice and the replication of dengue virus in various organs of mice after infection both by the intracerebral route and the peripheral routes with a hope that the mouse model might provide some parameters to represent the pathogenicity of the virus in further studies.

MATERIALS AND METHODS

Virus and infectivity assay method : Dengue virus type 2 (D−2), Tr 1751 strain in the 67th mouse brain passage level was used throughout the study. Stock virus was prepared from infected suckling mouse brain in a form of 10% suspension in phosphate buffer solution (pH 7.4) containing 0.75% bovine serum albumin and antibiotics (BPA). Infectivity titer of the stock virus was 10^7 plaque forming unit (PFU)/ml as assyed in BSC-1 cells by hemadsorption-negative plaque method described previously (Makino *et al*, 1975).

Mice: One day old, 3 day old, 5 day old and 4 week old mice of ICR strain randomly bred in our laboratory were used.

Virus inoculation: Stock virus was diluted in BPA and 0.02ml of virus was inoculated intracerebrally (ic), intramuscularly (im) from the hip muscle, intraperitoneally (ip) and subcutaneously (sc), respectively.

Sampling of the organs from infected mice : Infected mice were sacrificed at intervals after infection by bleeding. Nine species of organs, which are brain, thymus, heart, lung, liver, spleen, kidney and bone-marrow were taken. All samples except blood and brain were washed twice in phosphate buffer solution (PBS) to remove the blood in the organs and stored at −75°C until use. In some experiments, mice were anesthetized with ether and the blood in whole body was washed out by pouring enough PBS from the left ventricle by the use of 20 ml syringe followed by the removal of organs from the body.

To assay the virus titers in the organs, each pooled sample from 2 to 4 mice was ground in homogenizer with BPA to give 20% infected mouse tissue homogenates. Homogenates were centrifuged at 10,000 rpm for 30 min and the supernatants were assayed for virus content.

Assay of interferon-like substance: Test samples for interferon-like substance were obtained from the supernatant of 20% mouse tissue homogenates and partially purified by rendering them at pH 2.0 for 3 days and by centrifugation at 100,000 g for 1 hr after readjustment of pH to 7.4. The supernatant were serially diluted and incubated with L cell monolayers in the wells of tissue culture plate (Falcon No. 3040) overnight at 37°C. Interferon titer was estimated by the reciprocal of the dilution of the preparation which inhibits the cytopathic effect of challenging 100 tissue culture doses of vesicular stomatitis virus.

Transmission experiments of D-2 virus between mice and mosquitoes : Adult female mosquitoes of Bangkok strain of *Aedes aegypti* were allowed to feed on infected suckling mice of 5th day after infection. Each five engorged mosquitoes immediately after engorgement and the blood of infected mice exposed to mosquitoes were harvested to measure the virus content. Engorged mosquitoes were incubated at 28°C and at intervals, each 10 mosquitoes were harvested to examine the replication of the virus in mosquitoes. In the transmission study from infected mosquitoes to mice, uninfected 3 day old suckling mice were exposed to infected mosquitoes, followed by the observation of sickness of mice for 14 days. The mice were sacrificed on day 14 and the 10% brain suspension was again inoculated by the ic route to a litter of suckling mice to examine if the virus can be recovered from the original mice exposed to the biting of the infected mosquitoes.

RESULTS

1. Susceptibility of mice to D-2 virus by different ages and by different infection routes.

It is a well known fact that infection of mice with D-2 virus by the ic route is the most susceptible and only method to cause a death in mice. To examine whether or not the susceptibility of mice to D-2 virus is dependent on the age of host and the route of infection, one day old, 3 day old, 5 day old suckling mice and 4 week old mice were inoculated with D-2 virus by the im, ip and sc routes in addition to by the ic route. Ten mice were used per one dilution. As shown in Table 1 the virus uniformly caused a rapid fatal infection in the 4 different ages of mice when inoculated by the ic route. The LD_{50} varied from about 3 to 12 PFU of virus with increasing age of the mice. On the other hand, by the im, ip and sc routes, the susceptibility of mice decreased by 1/100 to 1/1000 and even in 1 day old mice, the LD_{50} was respectively 2.7×10^3, 3.0×10^3 and 7.6×10^2 PFU of the virus. Moreover, uniformly lethal infections were not observed in older mice even though larger doses of virus were inoculated.

2. Replication of D-2 virus in various organs of mice by intracerebral infection.

Since D-2 virus was found to cause a fatal infection by the peripheral routes as well as by the ic route in 1 day and 3 day old suckling mice, virus replication in various organs of mice was examined at intervals after ic and im infection. Preliminary experiments on the virus contents in organs of mice taken after the complete removal of blood by washing whole

Table 1. LD_{50} of dengue virus type 2 in mice by different routes of infection

Age of mice \ Route of infection	ic[a]	im[b]	ip[c]	sc[d]
	PFU			
1 day old	3.0	2.7×10^3	3.0×10^3	7.6×10^2
3 day old	3.0	2.8×10^4	4.8×10^4	NE[e]
5 day old	6.5	NT[f]	4.8×10^4	NE
4 week old	12.0	NT	NT	NT

a, b, c and d : Representation of intracerebral infection, intramuscular infection, intraperitoneal infection and subcutaneous infection, respectively.
e: LD_{50} could not be estimated because of the lack of uniformly lethal infection.
f: Not tested.

body blood vessels with sufficient PBS showed no significant differences from those of organs taken after simple bleeding of mice (Table 2). Therefore the latter method was employed for the determination of virus contents in organs hereafter. As shown in Table 3, in 1 day and 3 day old suckling mice, the virus began to replicate in brains from 2 days after infection of 50 LD_{50} of virus, in bone-marrow from day 3 and in all of 7 other organs including blood from 5 days after infection. The virus infectivity titer in blood in 1 day old suckling mice ranged from $10^{4.3}$ to $10^{4.6}$ PFU/ml and was higher than that of 3 day old suckling mice ranging from $10^{3.3}$ to $10^{2.9}$ PFU/ml. Although the virus seemed to replicate in other organs of 1 day old suckling mice slightly better than in those of 3 day old suckling mice, no significant differences of the virus replication was observed in these suckling mice.

On the other hand, in 5 day old suckling mice and 4 week old weanling mice, the virus replication was demonstrated only in brain and bone-marrow and the virus was scarcely recovered from other organs and blood (Table 4). From these observations, the susceptibility

Table 2. Comparison of virus contents in organs of infected suckling mice taken by different two methods

Organs \ Methods	Routine[a]	Washing[b]
blood	3.6[c]	NT[d]
brain	7.3	7.3
thymus	3.9	3.7
heart	1.8	trace
lung	2.4	2.1
liver	2.3	2.5
spleen	2.8	2.8
kidney	3.0	2.9
bone-marrow	4.3	4.2

Infected mice were sacrificed on day 6 after intracerebral inoculation with dengue virus type 2.

a : Organs were taken after bleeding of mice and then rinsed twice with PBS.

b : Organs were taken after removal of blood by washing whole body blood vessels with PBS.

c : Mean value of $\log_{10}$ PFU per ml of virus of 20% tissue homogenate of individual mouse. (4 to 5 determinations).

d : Not tested.

Table 3. Replication of dengue virus type 2 in various organs of 1 day old and 3 day old suckling mice after intracerebral infection with 50 LD_{50} of virus

Organs \ Days after inoculation / Age of mice	2		3		5		7	
	1 day old	3 day old	1 day old	3 day old	1 day old	3 day old	1 day old	3 day old
blood	UD[a]	UD	UD	UD	4.6	3.3	4.3	2.9
brain	3.2[b]	2.1	4.2	4.0	7.0	7.1	7.9	7.7
thymus	UD	UD	UD	UD	2.2	2.8	3.0	2.5
heart	UD	UD	UD	UD	2.9	2.0	2.4	2.7
lung	UD	UD	UD	UD	2.8	2.7	2.7	2.3
liver	UD	UD	UD	UD	3.1	2.8	4.1	3.4
spleen	UD	UD	UD	UD	2.7	2.5	4.3	3.8
kidney	UD	UD	UD	UD	2.5	2.4	3.1	3.8
bone-marrow	UD	UD	2.3	2.7	3.1	3.6	4.0	3.2

a : Undetectable.

b : Mean value of virus titer ($\log_{10}$ PFU per ml) of 20% tissue homogenates of 2 to 4 mice.

of mice to D-2 virus seemed to be significantly different between the mice within 3 days of age and the mice older than 5 days.

3. Replication of D-2 virus in various organs by intramuscular infection.

Although intramuscular infection with 10imLD$_{50}$ of the virus of 1 day old mice caused the uniformly lethal infections with longer incubation period than those by the ic route, infection of 3 day old mice resulted in the manifestation of sickness and death in approximately 80% to 90% of mice from day 6 to 9 after infection. In this experiment, 3 day old suckling mice were inoculated with 10imLD$_{50}$ of the virus from the hip muscle and the infected mice at day 5 and the mice manifesting the sickness from day 6 to 8 were sacrificed to examine the replication of the virus. Table 5 demonstrates the average virus titers in various organs of 3 mice in which virus contents were assayed individually. Virus titer in blood was almost the same as that of 3 day old mice by the ic infection and ranged from $10^{2.2}$ to $10^{3.2}$ PFU/ml. Virus contents in brain, thymus, heart, liver and bone-marrow was also similar to those of 3 day old mice by ic infection. However, the virus was not demonstrated in any organ until day 6 and even thereafter the virus was scarecely recovered from the lung and the spleen.

Table 4. Replication of dengue virus type 2 in 5 day old mice after intracerebral infection with 50 LD$_{50}$ of virus

Organs \ Days after infection	3	5	7	9
blood	UD[a]	UD	UD	UD
brain	2.5[b]	5.0	5.7	6.3
thymus	UD	UD	UD	trace
heart	UD	trace	UD	trace
lung	UD	UD	UD	trace
liver	UD	UD	UD	UD
spleen	UD	UD	UD	trace
kidney	UD	UD	UD	trace
bone-marrow	UD	2.3	4.6	5.4

a : Undetectable.
b : Mean value of virus titer ($\log_{10}$ PFU per ml) of 20% tissue homogenates of 2 mice.

4. Interferon production in various organ of mice infected with D-2 virus.

To examine the possibility that interferon production in the mice after infection with

Table 5. Replication of dengue virus type 2 in 3 day old suckling mice after intramusclar infection with 10 im LD$_{50}$ of virus

Organs \ Days after inoculation[a]	5	6	7	8
blood	UD[b]	2.3[c]	3.2	2.2
brain	UD	6.8	5.7	7.0
thymus	UD	UD	3.0	2.4
heart	UD	2.2	UD	2.5
lung	UD	trace	trace	trace
liver	UD	3.0	2.8	UD
spleen	UD	UD	trace	UD
kidney	UD	UD	trace	2.8
bone-marrow	trace	3.9	2.4	3.4

a : The mice of 5 days after inoculation showed no apparent change and the mice of 6,7 and 8 days after inoculation were manifesting the sickness.
b : Undetectable.
c : Mean value of virus titer ($\log_{10}$ PFU per ml) of 20% tissue homogenate of individual mouse organ.

D-2 virus reflects the virulence of virus and to know the involvement of interferon in the recovery from dengue virus infection in mice, interferon production in mouse organs was examined at intervals following virus infection by the ic route. Interferon was found only in brains after 5 days of infection and was not detectable in other organs. The kinetics of interferon production in brain in each age group of mice was shown in Table 6. The amount of interferon was lower in older mice and in every case, the interferon level was directly related to the amount of virus production and usually was first detectable when virus titer greater than 10^5 PFU/ml of brain suspension were reached.

5. Transmission of D-2 virus between mosquitoes and mice.

Since dengue viruses are transmitted by mosquitoes in nature, experimental transmission study was tried between adult female of *Ae. aegypti* and suckling mice. One day old and 3 day old suckling mice were rendered viremic by the ic infection of 50 LD_{50} of virus. On day 5 after infection, the mosquitoes were allowed to feed upon the mice. As shown in Table 7, the pooled blood of these mice was found to contain 2.5×10^4 PFU per ml of blood in 1 day old mice and 8.5×10^3 PFU in 3 day old mice respectively. Macroscopically engorged mosquitoes were found to feed 50 PFU in average of virus per individual mosquito in the first experiment and 17 PFU in the second experiment. Virus began to be detectable from mosquitoes after 9 days of incubation at 28°C, and on day 12 of incubation, virus titers of 7.0×10^3 and 1.2×10^2 PFU/mosquito were obtained respectively.

Table 6. Interferon production in brains of mice infected with dengue virus type 2 by intracerebral route

Age of mice \ Days after inoculation	2	3	5	7
1 day old	<10[a] (3.2)[b]	<10 (4.2)	120 (7.0)	180 (7.9)
3 day old	<10 (2.1)	<10 (4.1)	112 (7.1)	226 (7.7)
5 day old	<10 (1.4)	<10 (2.5)	28 (6.3)	56 (7.0)
4 week old	<10 (UD)[c]	<10 (2.7)	20 (5.0)	24 (5.9)

a : Interferon titers were expressed as the reciprocal of dilution of the preparations which inhibited the cytopathic effect of VSV more than 50% in L cells.

b : Numbers in parenthesis show mean value of virus titer ($\log_{10}$ PFU per ml) of 20 % homogenate of brain.

c : Undetectable.

Table 7. Transmission of dengue virus type 2 from viremic mice to *Aedes aegypti*

Experiment No.	Age of infected mice	Viremia titer (PFU/ml of blood)	Virus titer per mosquito immediately after engorgement(PFU)	Virus titer per mosquito after 12 days of incubation at 28°C (PFU)
1	1 day old	2.5×10^4	50	7.0×10^3
2	3 day old	8.5×10^3	17	1.2×10^2

The virus was shown to be transmitted to normal suckling mice by the bite of infected *Ae. aegypti*. Two groups of 2 day old mice bitten by one and two mosquitoes at 11th day after infection remained healthy for 14 days of incubation. However, the virus was found to persist in the brains of each half of mice since in the second passage of their brains, mice became sick 12 to 16 days later and subsequently, the D-2 virus was recovered (Data not shown). Further detailed experiments are necessary to examine the factors influencing the transmission and attack rate to mice from infected mosquitoes and the infection rate to the mosquitoes from viremic suckling mice.

DISCUSSION

In the present study, the susceptibility of mice to D-2 virus was studied by examining the interrelationships among the age of mice, the route of infection, the mortality and the degree of virus replication in various organs. The virus was inoculated by the ic, im, ip and sc routes into 1 day old, 3 day old, 5 day old and 4 week old mice. The results demonstrated that the mice younger than 3 days of age can be lethally infected even by the extraneural peripheral routes. In addition, the virus replicated in all 9 species of organs tested after infection by the ic route. Similarly, the virus was recovered from blood and other organs after the im infection in 3 day old mice except that the initiation of the virus replication was slower than that by the ic route and the virus was scarecely recovered from the lung and spleen.

Whereas, in the mice older than 5 days of age infected by ic route, the virus replicated only in brain and bone-marrow and the virus was not recovered from the blood despite of being no differences in the mortality from the mice younger than 3 days of age.

These results indicated that the age of mice is the important factor for the susceptibility of mice to D-2 virus. The susceptibility of the mice younger than 3 days of age differs significantly from that of the mice older than 5 days of age. The results also might imply that the pathogenesis of D-2 virus in mice by the im infection is not identical to that by the ic infection.

Virulent strains of viruses are considered, in general, to produce less interferon or to be more resistant to interferon than attenuated strains, but there are instances when this is not the case (Smith, 1972). In the present study, interferon production was only demonstrated in brain after the virus titer greater than 10^5 PFU/ml of brain suspension were reached. The amount of interferon was lower in older mice. The results obtained were essentially identical to those described by Cole and Wisseman (1969) in dengue virus infection in mice and Vilcek (1964) in sindbis virus infection in mice. It was suggested that in this system, the amount of interferon induced simply reflects the rate of virus replication and interferon production has little or no effect on the outcome of infection.

Little has been reported concerning the presence of viremia after infection of D-2 virus in small laboratory animals. The present study demonstrated the development of viremia in mice younger than 3 days of age after infection either by the ic route or the im route and

this was confirmed by the success of virus transmission to adult female *Ae. aegypti*. Although virus transmission from infected mosquitoes to mice resulted in an inapparent infection in the study and further studies are needed in various aspects, such an experimental combination of suckling mice and *Ae. aegypti* appeared to be possible to provide a laboratory animal model in which the virus transmission of dengue viruses can be studied.

Finally, the results of the study demonstrated several events in mice after infection with D-2 virus, however, at a given time, it is uncertain which of these events would be a good parameter reflecting the pathogenicity of the D-2 virus. Further comparative studies are being carried out in mice using fresh isolate, mouse brain adapted and tissue culture adapted strains of dengue virus.

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マウスにおけるデングウイルス感染

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デング出血熱やデングショック症候群がデングウイルス感染によって起こることはよく知られている．然し乍ら，これらの疾患の病原性やウイルス株による病毒性に関する研究を推進するためには実験動物モデルの確立が望まれている．マウスとデングウイルスの系による実験は報告されているもののなお基礎的な研究に多くの余地が残されていると考え，著者らはマウスとデングウイルス2型を用いて基本的な実験を行ない，次の如き結果を得た．

1．デングウイルス2型とマウスの日令差による感受性との関係においては，生後3日以内の哺乳マウスは生後5日以上のマウスに比較して非常に高い感受性を示した．即ち，a）3日令以内の哺乳マウスでは脳内接種と同じく末梢感染－筋肉内，腹腔内，皮下接種－によっても発症死亡した．然し5日令以上のマウスでは脳内接種によってのみ発症死亡した．b）3日令以内の哺乳マウスでは脳内接種及び筋肉内接種によって感染後ウイルス血症を確認することができたが，5日令以上のマウスにおいては検出することができなかった．c）ウイルスを脳内接種しマウス体内におけるウイルス増殖を経日的に検索したところ，3日令以内の哺乳マウスにおいては検査した9種類の臓器即ち，血液，脳，胸腺，心，肺，肝，脾，腎及び骨髄のすべてにウイルス増殖を認めたが，5日令以上のマウスでは脳及び骨髄にのみ検出されたに過ぎない．

2．インターフェロン様物質の産生についてはいずれの日令グループのマウスにおいても感染後脳内にのみ認められた．然しその産生はマウスの感染からの回復には関与しているとは考えられず，単にその産生量はウイルス増殖度合に関連しているに過ぎなかった．

3．ウイルス血症時の哺乳マウスからネッタイシマカ雌成虫へのウイルス伝播及び感染蚊から新生哺乳マウスへのウイルス伝播実験については，後者の伝播実験がマウスの不顕性感染にとどまり，発症することはなかったとは云え証明することが出来た．

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