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PATHOGENICITY OF NEGISHI VIRUS IN MICE CHARACTERIZED BY AGE OF SUSCEPTIBILITY, ROUTES OF INOCULATION AND GROWTH OF THE VIRUS IN TISSUES

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Pathogenicity of Negishi virus, a member of the tick borne encephalitis virus group isolated in Japan, was examined in mice. Weaning mice were highly susceptible to lethal infection of Negishi virus upon both intraperitoneal (ip) and subcutaneous (sc) inoculations, and less than 1.0 PFU per 0.1ml of virus was sufficient to kill 50% of the mice in both instances. On the other hand, weaning mice were relatively resistant to peroral (po) infection of the virus, and the lethal dose 50 was $10^{7.5}$PFU. Resistance of ageing mice was observed when the mice were inoculated with Negishi virus via the ip route. Negishi virus first appeared in the spleen of the mice inoculated via the sc route. The highest titer of Negishi virus ($10^{4.0}$ to $10^{5.0}$) was obtained in the brains of mice infected via the sc and ip routes. The virus was not detected in the blood during the course of infection. A relatively high titer of Negishi virus ($10^{3.0}$ to $10^{4.0}$) persisted in the small and large intestines of infected mice.

Key words : Negishi virus, tick-borne encephalitis virus, flavivirus, pathogenicity, infection

INTRODUCTION

Eighty kinds of tick borne arboviruses are registered in the Arbo Virus Catalouge of 1975.[3] Of these viruses, Russian spring-summer encephalitis virus, Omsk hemorrhagic fever virus and louping ill virus belong antigenically to the tick borne encephalitis complex of Flavivirus. Tick borne encephalitis is now endemic in ares of high latitude including the USSR, northern and eastern European countries and Canada.

Negishi virus was isolated in Japan by ANDO *et al.* in 1948[2] from a human with symptoms resembling Japanese encephalitis. Antigenic analysis revealed the virus to belong to the tick borne encephalitis virus group.[6] HIRONAKA[5] reported that mice are highly susceptible to intra-nasal and intra-testicular infection of Negishi virus. Beyond these, no further reports are available concerning the pathogenicity, ecology and vector of Negishi virus.

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The purpose of this study was to determine the pathogenicity of Negishi virus in mice by the following : 1) examination of susceptibility of mice to Negishi virus infection via different routes of inoculation ; 2) determination of the age of susceptibility of mice to Negishi virus ; and 3) estimation of growth and distribution of Negishi virus in various tissues of infected mice.

MATERIALS AND METHODS

Virus strain :

The Negishi virus used in this study was originally isolated by ANDO *et al.* in 1948[2] from a patient manifesting symptoms resembling Japanese encephalitis. The virus was kindly provided by Dr. A. OYA of the National Institute of Health in Japan and inoculated into the brains of suckling mice to prepare stock virus. Infected mouse brains were made into 10% or 50% suspensions in Dulbecco's phosphate buffered saline (PBS) containing 5% of fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The suspension was centrifuged at 10,000rpm and 4°C for 20 minutes. Supernatant was subdivided and stored at −85°C.

Cell culture and plaque formation :

The continuous BHK cell line used originated from a hamster kidney and was maintained in Eagle's minimum essential medium (Eagle's MEM Nissui, Japan) containing L-glutamine (0.292g/1), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and 5% fetal calf serum. One ml of trypsinized cells was seeded onto plastic culture plates containing 24 wells (Nunc, Denmark) at a cell concentration of 4×10^5/ml. The plates were then incubated at 37°C in 5% CO_2 for 24 hours. Next, the plates with a cell monolayer were inoculated with 0.1ml of virus suspension and incubated for 90 minutes. After incubation, all the wells were filled with 1ml of Eagle's MEM overlay medium containing 1.5% carboxymethyl cellulose, 3% tryptose phosphate broth, 1% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and L-glutamine (0.292g/1). After 4 days of incubation, the culture fluid in the plates was removed, and the plates were fixed in a solution containing 2.5% potassium dichromate, 5% glacial acetic acid and 5% formalin for 30 minutes. The stained plates were then washed with tap water and counted for plaque number.

Detection of virus in the tissues of infected mouse :

An infected mouse was anesthetized by ether, and tissues from the brain, spleen, kidney, liver, prescapular lymph node, small intestine (duodenum and jejunum) and large intestine (cecum and colon) and blood were obtained. Serum was separated from the blood. Materials obtained were stored at −85°C. All the tissues except those from the small and large intestines were weighed and grounded into 10% suspension with diluent in mortars. The suspension was centrifuged at 10,000rpm and at 4°C for 20 minutes, and the supernatant was stored at −85°C. The small and large intestines were cut open to remove their contents and then washed with diluent. After washing, supernatant of

10% suspension of the intestines was prepared as described above and stored at $-85°C$. The tissues and serum were diluted at ten fold, and 0.1ml of each dilution was inoculated onto a BHK cell monolayer. Virus titers were obtained by plaque counts.

Oral administration of virus :

A mouse stomach feeding tube made of stainless steel (70mm × 1mm) was used for oral administration of the virus. After depriving the mouse of drinking water for one day, the tip of the stomach feeding tube containing the virus was placed near the mouth of the mouse to facilitate self obtainment of the virus suspension.

Results

Susceptibility of mice to Negishi virus infection was compared for the three different ways of inoculation including ip, sc and po routes (Table 1). Weaning mice (2 to 3 weeks) proved to be very susceptible to the infection by either the ip or sc routes. In these mice, LD_{50} values of undiluted inoculum were 10 fold higher than the titer of an undiluted inoculum expressed as PFU, and one LD_{50} was less than one PFU in both instances. Three-week-old mice were relatively resistant to po infection of the virus and died only at a high dose of the virus. One LD_{50} of po infection corresponded to $10^{7.5}$PFU. Resistance to the viral infection with ageing of mice was clear in the ip route of infection. Lower LD_{50} values of the undiluted inoculum in the ip route were expressed in the eight-week-old mice ($10^{2.6}$) than in the 2-week-old mice ($10^{7.0}$). And some of the 8-week-old mice which survived after subcutaneous virus inoculation had detectable neutralizing antibodies (data not shown).

Growth and distribution of Negishi virus were examined in three-week-old mice after subcutaneous infection (Table 2). Fifteen mice were inoculated with 0.1ml of the virus suspension containing $10^{4.3}$PFU and were sacrificed every day for virus titration in each tissue until day 7. The virus was first detected in the spleen on day 2 and was consistently found during the observation period. The highest titer of virus (higher than $10^{4.0}$PFU) was obtained in brains after 5 days of inoculation. Relatively high titers of the virus were present in small and large intestines. The lymph nodes and kidney also contained the virus after 6 days of inoculation. The virus was not detected in the serum and liver.

Distribution of Negishi virus was examined in one three-week-old mouse after oral infection. The mouse was administered the virus of $10^{8.5}$PFU/0.1ml and sacrificed after 6 days ; Table 3 shows the virus titers in the serum and tissues. The titer of virus was the highest in the brain ($10^{5.0}$PFU/ml) followed by the large intestine ($10^{4.6}$), kidney ($10^{4.3}$), small intestine ($10^{3.5}$), spleen ($10^{3.5}$), and lymph nodes ($10^{2.6}$). Distribution pattern of the virus in the peroral infection was essentially the same as that of the subcutaneous infection on day 6, although the virus titer was higher in the peroral infection than in the subcutaneous one.

TABLE 1 *Susceptibility of mice to Negishi virus infection after three different routes of inoculation*

ROUTE OF INOCULATION	AGE OF MICE IN WEEK	TITER OF[1] UNDILUTED VIRUS INOCULUM	NO. OF MICE DEAD/TOTAL VERSUS DILUTION OF VIRUS					LD_{50} OF[2] UNDILUTED INOCULUM	PFU EQUIVALENT TO ONE LD_{50}
			undiluted	10^{-2}	10^{-4}	10^{-6}	10^{-8}		
ip	2	6.0	5／5	5／5	5／5	5／5	0／5	7.0	−1
	4	6.0	5／5	5／5	5／5	0／5	0／5	5.0	1
	8	6.0	3／5	2／5	3／5	1／5	0／5	2.6	3.4
sc	3	6.0	2／3	3／3	3／3	3／3	0／3	7.0	−1
	8	6.0	2／3	3／3	2／3	2／3	0／3	5.2	0.8
po	3	8.5	8／8	0／8	−[4]	−	−	1.0	7.5

1) Titer was expressed as log_{10} PFU per 0.1 ml.
2) Lethal dose 50% (LD_{50}) of undiluted inoculum was calculated from death rates of mice at each dilution (log_{10}).
3) PFU (log_{10}) equivalent to one LD_{50} was calculated from PFU of undiluted virus inoculum and LD_{50} of undiluted inoculum in mice.
4) −, Not determined

TABLE 2 *Growth and distribution of Negishi virus in 3-weeks-old mice after subcutaneous inoculation*

DAYS AFTER INOCULATION	MOUSE CODE NO.	VIRUS TITER IN[1]							
		Serum	Brain	Spleen	Kidney	Liver	Lymph node	Small intestine	Large intestine
1	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
2	3	0	0	2.5	0	0	0	0	0
	4	0	0	2.9	0	0	0	0	0
3	5	0	0	0	0	0	0	0	0
	6	0	0	2.0	0	0	0	0	0
4	7	0	0	2.0	0	0	0	0	0
	8	0	0	NT[2]	0	0	0	1.3	0
5	9	0	4.0	2.3	0	0	0	1.6	0
	10	0	4.0	2.9	0	0	0	3.8	3.0
6	11	0	5.0	2.0	1.0	0	0	2.0	3.0
	12	0	0	2.0	1.8	0	1.8	3.8	3.0
7	13	0	5.0	2.0	1.2	0	3.4	3.3	4.4
	14	0	5.2	0	2.4	0	2.8	3.2	3.0

1) Titer as $\log_{10}$ PFU per 1 ml.
2) NT, not tested.

TABLE 3 *Distribution of Negishi virus in one 3-week-old mouse on day 6 after oral infection*

ORGAN	VIRUS TITER[1)]
Serum	0
Brain	5.0
Spleen	3.3
Kidney	4.3
Liver	0
Lymph node	2.6
Small intestine	3.6
Large intestine	4.6

1) Titer was expressed as $\log_{10}$ PFU per 1 ml.

DISCUSSION

The present study demonstrated that age and route of inoculation affected the lethality of Negishi virus infection in mice. Weaning mice were very susceptible to ip and sc inoculation, and one LD_{50} value was less than 1.0 PFU in both instances. The same age mice were relatively resistant to peroral infection. Adult mice were found to be less susceptible to lethal infection of Negishi virus than the weaning mice in the ip route of infection. Some of the adult mice which recovered from infection had detectable neutralizing antibody, which showed that an inapparent infection had occurred in these mice. This may have been due to the development of a mature immune defense system and/or a so-called blood-brain barrier.[1,8] Resistance with ageing of mice to lethal infection was also noticed in another flavivirus.[4]

Peroral infection of tick borne encephalitis virus was reported in humans via infected goat milk and also in experimentally infected mice[7,9] ; therefore, infection of Negishi virus via peroral route deserves careful attention. In the present study, however, peroral infection of the mice to Negishi virus required a high dose of virus. Further experiments with other groups of flavivirus may be neccessary to conclude that the peroral infection of Negishi virus was due to the character of the tick borne encephalitis virus complex.

The earliest appearance of the virus in the spleen was noticed in infected mice after subcutaneous infection while the virus was not detected in the serum during the observation period. HIRONAKA[5] reported that the viremia of Negishi virus occurred after six hours of intratesticular inoculation in mice. He detected the virus in suckling mice which had been intracerebrally inoculated. The absence of viremia in this study may be due to low sensitivity of the tissue culture method to detect the virus as shown in Table

1. The high titer of virus persisted in the small and large intestines until day 8 in the mice inoculated subcutaneously ; this was also true in the mice administered perorally. High affinity of the virus to the intestines may be an important character of the tick borne encephalitis virus complex. The virus was not detected in the liver. The supernatant of liver suspension had a weak cytotoxicity to BHK cells used for virus assay. This result may indicate that a more sensitive method is needed to detect the virus in the liver.

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