DENGUE-3 (16562) PGMK 33 VACCINE : NEUROVIRULENCE, VIREMIA AND IMMUNE RESPONSES IN *MACACA FASCICULARIS*

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Abstract. Investigation of monkey neurovirulence of dengue-3 viruses (DEN-3, 16562) was undertaken to provide an evaluation of the relative safety of virus strain attenuated for potential use of live virus vaccine. Ten flavivirus-negative, cynomolgus monkeys *(Macaca fascicularis)* were used in the test. The animals were inoculated intrathalamically, intraspinally and intramuscularly with DEN-3 PGMK 33 attenuated live virus vaccine (6 monkeys) : parent virus (2) and control cell cuture fluid (2). Blood samples were collected on days 0, 1, 2, 4, 6, 8, 10, 12 and 21 for virus isolation and days 0 and 21 or 22 for serologic testing. One monkey with DEN-3 (16562) PGMK 33 candidate vaccine had detectable viremia on day 10. By day 21, all recipients of PGMK 33 and both monkeys with DEN-3 parent virus developed serum neutralizing antibodies to DEN-3 titers ranged from 56-320. The monkeys showed no evidence of illness and none died of dengue infection. Histopathological examination of tissue collected on day 21 or 22 revealed only minimal neurovirulence lesions as scored by the routine grading system. No differences were observed between the DEN-3 parent and vaccine viruses and it is concluded that neither virus is neurovirulent for cynomolgus monkeys.

INTRODUCTION

Monkey neurovirulence testing is a standard method for determining the safety of any live attenuated virus vaccine. The tests were performed according to the Code of Federal Regulation (CFR) USA 1982, Food and Drugs (FDA), Chapter I, Subchapter F-Biologics Part, 21 CFR 600. During the development of attenuated vaccine against dengue-2 virus infection, a decision was made at the same time to test the candidate vaccine in cynomolgus monkeys *(Macaca fascicularis)* in addition to rhesus monkeys *(M. mulatta)* (Angsubhakorn *et al,* 1986, 1987a,b) desired to meet US-FDA minimum safety requirements according to the World Health Organization (WHO) Dengue Vaccine Peer Review Committee recommendation. The tests were conducted under similar conditions to those prescribed for safety testing of vaccine in rhesus monkey (Nathanson *et al,* 1965).

Our first study in DEN-2 (strain 16681-PDK 53) vaccine attenuated by passage in primary dog kidney cells in cynomolgus monkeys (Angsubhakorn *et al,* 1986) showed that there was an acceptable minimal neurovirulence, and the vaccine has been given a clinical trial in man (Bhamarapravati *et al,* 1987). Since then, DEN-1 (Strain 16007-PDK 43) and DEN-4 (Strain 1036-PDK 48) virus vaccines have subsequently been tested in both species of monkeys (Angsubhakorn *et al,* 1987 a, b, 1988). No significant neurovirulence was observed with DEN-1 and DEN-4 attenuated by passage in PDK cells or their parent viruses in either rhesus or cynomolgus monkeys. These vaccines are now ready for further clinical trials.

In order to establish the cynomolgus as an acceptable model for neurovirulence testing of all dengue vaccine serotypes. In the present study, the last of this series, we describe the neurovirulence, viremia and immune responses by DEN-3 (Strain 16562 – PGMK 33) vaccine attenuated by passage in primary green monkeys kidney cells in cynomolgus monkeys.

MATERIALS AND METHODS

Animals

The cynomolgus monkey experiment was performed in the Toxicopathology Research Laboratory, Department of Pathobiology, Faculty of Science,

Mahidol University, Bangkok, Thailand. Eight animals were obtained from local traders in Thailand and two animals were bred in our laboratory animal facilities. The animals of both sexes, weighing 1.0-2.8 kg at the time of inoculation, were maintained individually in air-conditioned (24-25°C), mosquito free facilities and fed monkey pellets, fresh fruits and water *ad libitum*.

Viruses

DEN-3 virus, strain 16562, was isolated from a dengue hemorrhagic fever (DHF) patient in Bangkok. The virus has been successfully passaged in primary green monkey kidney (PGMK) cells in the Center for Vaccine Development, Mahidol University. At PGMK passage 33°, the virus formed large and small plaques, caused cytopathic effects (CPE) in LLC-MK_2 cells, and was not temperature restricted. Only small plaque formation was observed at PGMK 33 and higher passages. It appears that a significant change in the biological characteristics of DEN-3 (16562) occurred between PGMK 30 and 33. The PGMK 33 was finally chosen for clinical lot production.

Neurovirulence studies were done on production seed, parent DEN-3 (16562) and virus-free cell culture fluids (PGMK No. 8, Sublot 8). Parent and candidate DEN-2 vaccine viruses from blood, brain and spinal cord were kept in dry-ice and isolated at the Center for Vaccine Development, were inoculated intrathoracically into *Toxorhynchites amboinensis* mosquitos (Rosen and Gumbler, 1974) and identified by plaque-count titrations in LLC-MK_2 cells using single overlay and immunofluorescent techniques (Yuill *et al,* 1968).

Serum samples were examined for hemagglutination-inhibiting (HI) antibody against DEN 1-4 antigens and Japanese encephalitis (JE) virus (Clarke and Casal, 1985) and plaque reduction neutralization titers (PRNT) were measured as previously described (Russell *et al,* 1961).

Experimental design

Ten cynomolgus monkeys, tuberculin negative and free of HI and PRNT antibodies against DEN-1-4 and JE viruses, were anesthetized with ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, New York 13211) and phenobarbitone sodium (Nembutal, Abbott Laboratories, North Chicago, IL 60064). The subjects were fasted 17 hours prior to the experiment.

Two animals received virus-free cell culture fluids, 6 received candidate vaccine (1.8×10^5 plaque forming units, pfu/ml) and two received parental DEN-3 (1.1×10^4 pfu/ml). Inoculation was performed by combined intrathalamic (2×0.5 ml), intralumbar spinal (0.5 ml) and intramuscular (0.1 ml) of appropiate material into each monkey in each group. Blood samples were collected on day 0, 1, 2, 4, 6, 8, 10, 12 and 21 for virus isolation and on day 0 and 21 or 22 for serological testing. Twenty-one to 22 days after inoculation all monkeys were anesthetized, euthanized, and perfused through the left ventricle of the heart with 200-300 ml phosphate buffered saline (PBS) followed by 300-500 ml of 10% buffered formalin solution mixed with 5% glacial acetic acid. The entire brain and spinal cord were removed, examined, weighed and immersed in 10% buffered formalin solution.

Histopathological studies

One week after immersion in 10% buffered formalin solution, the specimens were trimmed to obtain representative sections of cerebral cortex (frontal, temporal, parietal and occipital lobes), cerebellum, midbrain, pons, medulla oblongata, and all 3 levels of the spinal cords (cervical, thoracic and lumbar), basal ganglia, thalami and choroid plexus. The grading system used was as described previously (Angsubhakorn *et al,* 1987 a,b); in brief, grades 1 and 2 were based on inflammatory lesions while neuronal destruction was the primary criterion for grades 3 and 4.

RESULTS

Virus and immune responses

Day of viremia and antibody response in cynomolgus monkeys are shown in Table 1. Detectable HI and PRNT antibody responses were observed in all 6 monkeys inoculated with candidate vaccine (PGMK 33) and 2 monkeys inoculated with parent virus (16562). All 6 monkeys receiving the DEN-3 vaccine developed HI (range 20-320) and PRNT antibody titers (range 56-320) to DEN-3. In addition, these animals developed cross-reacting antibodies to DEN-1, 2 and

Table 1

The day of antibody response in cynomolgus monkeys following inoculation with DEN-3 parental virus (16562) and candidate vaccine (DEN-3 16562 PGMK 33).

Inoculum	Monkey No.	Sex	Virus dose	Day of viremia	Reciprocal titers of dengue and Japanese encephalitis viruses									
					HI					PRNT				
					Dengue				JE	Dengue				JE
					1	2	3	4		1	2	3	4	
Control fluid	MU 90-2	m		0	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
	MU 90-3	m		0	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
DEN-3 virus	MU 90-7	f	1.1×10^4	0	10	10	40	20	20	14	12	86	38	25
	MU 90-8	m	1.1×10^4	0	10	20	80	40	20	22	40	50	80	42
DEN-3 vaccine	MU 90-4	f	1.8×10^5	0	20	20	80	40	20	46	52	210	80	52
	MU 90-5	f	1.8×10^5	10	10	10	80	10	10	23	14	160	22	29
	MU 90-6	m	1.8×10^5	0	20	10	320	20	20	23	24	320	52	40
	MU 90-15	m	1.8×10^5	0	20	10	40	20	20	22	23	90	44	34
	MU 90-16	m	1.8×10^5	0	+ 10	< 10	40	+ 10	+ 10	20	< 10	98	21	22
	MU 90-17	m	1.8×10^5	0	10	+ 10	20	10	10	23	15	56	13	13

Table 2

Distribution of neurovirulence lesions in monkeys caused by DEN-3 viruses.

CNS sections	No. animals with lesions									
	Control fluid		DEN-3 parental virus		DEN-3 vaccine virus (PGMK 33)					
	MU 90-2	MU 90-3	MU 90-7	MU 90-8	MU 90-4	MU 90-5	MU 90-6	MU 90-15	MU 90-16	MU 90-17
Frontal lobe				NR	V-1					NR
Temporal lobe	NR	V-1		NR		V-1				NR
Parietal lobe		NR		V-1				NR	NT	NR
Occipital lobe		V-1		NR				NR		NR
Basal ganglia				NT		V-1		V-1		V-1
Thalamus	NT		V-1, NT		NT	NT		NT	V-1, NT	NT
Cerebellum				V-1						NR
Midbrain			V-1	V-1		NT	V-1		NR	V-1
Pons				NR				V-1	NR	V-1
Medulla oblongata								V-1	V-1	
Cervical sp cd									V-1	
Thoracic sp cd	NR					V-1		NR	V-1	
Lumbar sp cd	NR		V-1, NT		NT	V-1, NT	NT	NT	V-1, NT	V-1

sp cd = Spinal cord; NT = Needle tract lesions; NR = Non-specific response; V-1 = Minimal neurovirulence lesions.

Table 3

Summary of pathological changes caused by DEN-3 viruses in *Macaca fascicularis.*

Histopathological findings	Types of inoculum		
	Control fluid	DEN-3 vaccine	DEN-3 parental virus
No. of animals used	2	6	2
Total CNS sites examined	26	78	26
No. CNS sites with NVL	2 (8)*	18 (23)	6 (23)
No. with V-1 lesions	2 (8)	18 (23)	6 (23)
No. with NR	4 (15)	10 (13)	4 (15)
No. animals with NT in:			
Brain	1	7	2
Spinal cord	0	5	1

* Percentage with NV lesions shown in parentheses; NVL : Neurovirulence lesions: NT : Needle tract lesions; NR : Non-specific responses; V-1 : Minimal neurovirulence lesions.

4 and JE (range 10-40 for HI and 13 to 80 for PRNT), except for monkey no. MU 90-16 which had no detectable HI antibodies to DEN-2. By day 21, two monkeys (MU 90-7 and MU 90-8) with DEN-3 parent virus also developed serum PRNT antibodies that cross reacted with all 4 dengue serotypes and JE. Homologous DEN-3 PRNT titers ranged from 50-86 while heterologous flavivirus titers were 12-80. The cross reaction of DEN-3 with DEN-4, JE seemed to be higher than any other serotypes. No virus was isolated from brain and spinal cord for any monkey while one monkey (MU 90-5) developed viremia on day 10 when inoculated with 1.8×10^5 pfu/ml of DEN-3 candidate vaccine virus on day 10.

Histological findings

The predominant sites for occurrence of neurovirulence lesion (NVL) in cynomolgus monkeys for DEN-3 virus were midbrain, basal ganglia and lumbar spinal cord (Table 2).

Histopathologic neurovirulence lesions are grouped and summarized in Table 3. Both parent DEN-3 and the DEN-3 vaccine candidates were well tolerated by the monkeys used in this neurovirulence study. Animals inoculated with the DEN-3 vaccine candidate virus developed neurovirulence-type lesions which were graded as minimal (V-1) in severity, similar to minimal severity lesions seen in monkeys inoculated with the parent strain virus.

One, 7 and 1 needle track lesions were found in brains of monkeys treated with the control, vaccine and wild type of DEN-3 virus, respectively (Fig 1), while 0, 5 and 1 needle track lesions were found in their spinal cords (Fig 2). There was no clearcut evidence of neuronal destruction with either parent or vaccine candidate viruses of DEN-3. The neurovirulence lesions were generally characterized by minimal perivascular lymphoid cells infiltration and occasional glial (histiocytic) cell foci in the neuropil (Fig 3). One monkey inoculated with noninfected fluids had 2 lesions in the brain which met the criteria for a minimal (V-1) neurovirulence lesion. These lesions consisted of occasional perivascular lymphoid foci and glial nodules in the grey and white matter of the temporal and occipital lobes.

DISCUSSION

Both parent DEN-3 and the DEN-3 vaccine candidates were well tolerated by the cynomolgus monkeys used in this study. Animals inoculated with DEN-3 vaccine candidate virus developed neurovirulence lesions (NVL) which were graded as minimal (V-1) in severity, similar to minimal severity lesions seen in monkeys inoculated with the parental strain virus. There were no mild (V-2), moderate (V-3) or severe (V-4) NVL in any monkey inoculated

Fig 1– Right thalamus of a cynomolgus monkey inoculated with DEN-3 (16562), PGMK 33 virus showing a needle track lesion.

Fig 2– A portion of right lumbar spinal cord of a cynomolgus monkey inoculated with DEN-3 (16562) PGMK 33 virus showing a needle track lesion.

with either the parent or vaccine candidate strain of DEN-3 virus. With both strains of virus lesions tended to occur frequently in the frontal, temporal, and parietal lobes of the cerebrum, thalamus, pons, cerebellum, medulla oblongata, and the cervical and thoracic levels of the spinal cord. The predominant sites for NVL were midbrain, basal ganglia and lumbar spinal cord. There was no clearcut evidence of neuronal destruction with either the parental or vaccine candidate viruses of DEN-3. The NVL were generally characterized by minimal perivascular cuffs of lymphoid cells and occasional glial (histiocytic) cell foci in the neuropil. The distribution of these lesions was similar to that noted in neurovirulence tests of other dengue vaccines, *ie* lesions were most frequently located in the white matter of cerebrum, substantia nigra, basal ganglia, grey matter of the brain stem and the spinal column (Angsubhakorn *et al,* 1986, 1987a,b, 1988).

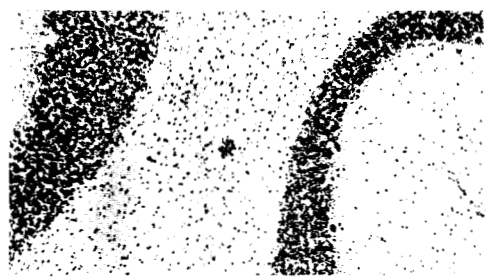

Fig 3– Cerebellum of a cynomolgus monkey inoculated with DEN-3 (16562) parental virus showing a glial nodule in the molecular layer, interpreted as a minimal neurovirulence lesion (V-1).

The nonspecific responses observed in all groups of monkeys, including those inoculated with the uninfected control fluids, were typical of those which occurred following various types of injections into the central nervous system. These nonspecific responses occurred with equal incidence in the monkeys inoculated with the DEN-3 vaccine candidate, noninfected control fluids, perhaps owing to the presence of greater innate nonspecific antigenicity or toxic cell degradation products in the infected cultures. Because nonspecific responses can occur following a wide variety of stimuli or injuries to the central nervous system, these were not interpreted as indicators of neurovirulence.

One monkey inoculated with noninfected control fluids had NVL in the brain which met the criteria for a minimal (V-1) neurovirulence lesions. These lesions consisted of occasional perivascular lymphoid foci and glial nodules in the grey and white matter of temporal and occipital lobes. This observation confirms the conclusion that the presence of lymphoid cuffs is not specific or definitive evidence of neurovirulence, but must be supported by the total pathologic picture.

In conclusion, firstly, neurovirulence lesions occurred in all cynomolgus monkeys inoculated with the DEN-3 vaccine candidate virus used in this study: these were of minimal (V-1) severity, nondestructive, and probably of little biological significance in regard to the safety of this vaccine. Secondly, the cynomolgus monkey *(M. fascicularis)* appears to be a good host animal species for neurovirulence testing, and probably responds with sensitivity similar to that observed in neurovirulence tests of other dengue virus vaccines conducted in rhesus monkeys (*Macaca mulata*) (Angsubhakorn *et al,* 1987 a,b, 1988). Thirdly, DEN-3 vaccine candidate virus tested in this study possessed relatively low neurovirulence potential and appears acceptably safe for continued development.

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