THE NEUROVIRULENCE OF FLAVIVIRUSES IN CRAB-EATING MONKEYS (*MACACA FASCICULARIS*)

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INTRODUCTION

Presently, accepted experimental protocol requires that the biological activity of attenuated dengue vaccines be neurovirulence tested in non-human primates, usually *Macaca mulatta* (rhesus monkeys). Rhesus monkey neurovirulence has proved to be an important test for attenuation of laboratory-modified strains of arboviruses developed as candidates for human vaccination. This test continues to remain the best and the final test of safety, similar to yellow fever vaccine (WHO, 1959) and is performed on different monovalent bulk fluids. Long-tailed macaques (Syn. Malayan, Philippines or Java monkeys) or popularly called crab-eating monkeys (*Macaca fascicularis*, *M. iris*) or Ling-Sa-Mae in Thai language, are small brown monkeys, with pale underparts and often prominent whitish hairs on the face. This primate is also much more prevalent than the rhesus in Thailand.

This study was designed to determine the feasibility of using this crab-eating macaque for neurovirulence testing of virus vaccines with potential for use in humans. An additional objective was to test the neurovirulence of an attenuated dengue-2 (DEN-2) vaccine. In order to establish points of reference in regard to the response of *M. fascicularis* to viruses with known propensity for neurovirulence, groups were also allocated for inoculation with yellow fever (YF) vaccine and with Japanese encephalitis (JE) virus.

MATERIALS AND METHODS

Animals: Eighteen male and female, 1-2 year crab-eating monkeys were born in the colony at Department of Veterinary Medicine, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Their parents originated in Peninsular Malaysia. All monkeys were housed in groups of 12-22 in large gang cases in an indoor facility. During this study, each animal was maintained in individual cage measuring $70 \times 62 \times 70$ cm^3. The subjects were fasted 17 hr prior the experiment. Pretreatment sera were screened for hemagglutination-inhibition (HAI) antibodies using the methods previously described (Clarke and Cassal, 1958). Sera were negative, at a level of 1 in 10, for DEN 1-4 and JE.

Viruses: DEN-2 (16681) parental virus, PGMK-5 isolated from a shock fatal case of dengue hemorrhagic fever (1×10^5 pfu/ml); DEN-2 (16681) PDK 53 vaccine (1×10^5 pfu/ml); and JE-virus, human strain, AP-61-1), C6/36-1 cell supernatant (1×10^5 pfu/ml) were used in this experiment. YF-vaccine, live BP 17D strain containing 1,000 mouse LD_{50} units (Arivax, The Wellcome Foundation Ltd., London) was purchased from Department of Communicable Diseases, Ministry

of Public Health. The final titers for DEN-2 parental virus inoculum in LLC-MK_2 cells were 3.7×10^5, 4.8×10^5 and 5.2×10^5 pfu/ml and titers for DEN-2 (16681) PDK 53 were 5.3×10^4, 6.2×10^4 (2), 8.0×10^4, 8.7×10^4 and 1.3×10^4 pfu/ml.

Monkey inoculation: A total of 18 crab-eating monkeys were randomized into the following groups: DEN-2 vaccine (6), Control culture fluid (3), DEN-2 parental virus (3), YF-vaccine (3) and JE-virus (3). Intraspinal inoculation: Each monkey was placed in lateral recumbency. A 3/4 inch, 27-gauge needle was inserted through the intervertebral space between the 1st and 2nd lumbar vertebrae and into the spinal cord until a "Jerk" reaction was observed in one of the legs. Inoculum (0.5 ml) was injected during a 10 second interval.

Intrathalamic inoculation: Each monkey was placed on the same surgical table in ventral recumbency. The head was supported on a fabricated wooden chin-rest. A template with holes drilled laterally an equal distance from the template midline (0.65 mm between holes), was firmly held on the calvarium with its anterior portion on the temporal suture and its midline on the parietal suture. Using a No. 2 drill bit (S.S White Dental Products International, PA. 19102) attached to a triple section arm of an electric dental drill (Buffalo Dental MFG, Co., Inc. Syossoset, NY. 11791), two holes were made, one through each side of the calvarium, at sites corresponding to the previously drilled holes in the positioned template. A 1¼ inch, 27-gauge needle was passed through the template hole into the brain until the needle hub contacted the upper surface of the template. During a 10 second interval, 0.5 ml of inoculum was injected slowly and steadily into each side of brain.

Intramuscular injection: An intramuscular injection of 1.0 ml of inoculum was given deep in the thigh muscle of the leg, caudal to the femur.

Clinical observation: Monkeys were returned to the proper cage and observed periodically until recovered from the anesthesia. The animals were followed from 19-20 days after inoculations, except that animals prostrated by encephalitis were sacrificed.

Necropsy of monkeys: The monkeys were anesthetized with ketamine hydrochloride and sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, 60064). The heart was surgically exposed through a saggital incision at the left lateral edge of the sternum. Thirty five ml of blood via cardiac puncture were taken from the right ventricle. The blood sample was divided and used for serum chemistry analysis, HAI and PRNT titers, and complete blood count. The results are reported elsewhere (Angsubhkorn *et al.*, 1986a).

The brain was perfused through the left ventricle of heart with 100 ml phosphate buffered saline (PBS), followed immediately by 100 ml of 10% buffered formalin mixed with 0.5% acetic acid.

The remaining spinal cord was exposed by complete dorsal laminectomy. Based on landmarks, the injection site was approximated and marked with silk ligature attached to the meninges. The brain and spinal cord were removed and placed in a gauze, tied with a long-string forming a sack, suspended in a wide-mouthed Mason jar filled with 10% bufferred formalin, and kept for histopathologic study. The skull of each monkey was labelled and kept for allocation of the needle point of entry of each cerebral hemisphere. All organs were weighed and recorded, and selected organs were stored in 10% bufferred formalin.

Histopathological studies

The specimens were trimmed, after at least 7 days of immersion fixation, to obtain sections of cerebral cortex (frontal, temporal perietal and occipital regions), cerebellum, midbrain, pons, medulla oblongata, spinal cord (cervical, thoracic and lumbar regions), basal ganglia, thalami, needle tracks and choroid plexus. Tissues were paraffin embeded, sectioned on a microtome, mounted on glass slides, stained with hematoxylin and eosin and examined microscopically. A modification of the grade-system, as described by Nathanson *et al.*, (1965), and Vickers (1982) was applied to the various CNS tissues. Briefly, the histopathological criteria used for each of the grades assigned are summarized in Table 1.

Table 1

Histopathological criteria for neurovirulence lesions.

CNS lesions	Description
V-1	**Minimal:** A few (usually <3 in a structure or region) thin cuffs of lymphocytes, usually only 1 or 2 cells thick, were seen in the Virchow-Robins spaces of blood vessels. If focal infiltration of macrophages (glial nodules) were present these occurred at a rate of <1 per microscopic (10x) power field (1pf). There was usually no evidence of neuronal degeneration or necrosis.
V-2	**Mild :** Perivascular lymphoid cuffing occurred more frequently, as many as 6 in a structure or region. Several glial nodules, from 2-6 per lpf, might be seen in 1 lpf, or more diffusely throughout the section If there was evidence of degeneration or necrosis, it involved < 10% of the neurons in a nucleus, structure, or region.
V-3	**Moderate :** Perivascular lymphoid cuffing occurred more frequently, affecting < 50% of the vessels in a structure or region, and was frequently composed of thick cuffs, > 6 cells thick. Glial nodules were abundant, from 7-12 per lpf. Evidence of neuronal degeneration or necrosis was seen in 11-60% of the neurons in a nucleus, structure, or region.
V-4	**Severe :** Perivascular lymphoid cuffing affected > 51% of the vessels in a structure or region and consisted of many thick cuffs. Glial nodules occurred very frequently with > 13 in selected 1 lpf. Neuronal degeneration or necrosis was evident > 61% of the neurons in a nucleus, structure or region.
NT	**Needle track :** These consisted of elongate, round, or irregular zone of malacia, hemorrhage, hemoglobin breakdown pigment, and foamy macrophage infiltration.
NR	**Nonspecific respones:** Small perivascular cuffs or infiltrations of lymphocytes in the meninges, choroid plexus, or immediate perivascular neuropil, when not accompanied by destructive local changes or other evidence of neurovirulence in a given section were interpreted as a nonspecific response to the process of inoculation.

RESULTS

There were clinical signs ranging from weakness to partial paralysis of either the left or right legs. JE-virus-inoculated monkeys became moribund and were sacrificed before termination of the usual 19-20 days. There were 14 monkeys in this series that had definite histological evidence of needle track lesions confined to the thalamus. Furthermore, 12 of 18 monkeys had definite histological evidence of inoculation lesions confined to the lumbar spinal cord.

Histopathological results are summarized in Tables 2 and 3. The monkeys used in this

Table 2

Distribution of neurovirulence lesions in crab-eating monkeys by flaviviruses.

CNS division	No. animals with neurovirulence lesions:						
	Control fluid (3)*	DEN-2 parental virus (3)	DEN-2 vaccine virus (6)	JE virus (3)		YF vaccine (3)	
	V-1	V-1	V-1	V-1	V-2	V-1	V-2
Frontal lobe				2		3	
Temporal lobe				2	1	3	
Parietal lobe				2	1	2	
Occipital lobe				2		2	
Basal ganglia		1	1	2	1	1	2
Thalamus	1		2	1	2	2	1
Cerebellum			1	2		1	1
Midbrain		1	1	1	2	3	
Pons			1	2	1	3	
Medulla oblongata		1		1	2	3	
Cervical spinal cord	1	1		1	2	3	
Thoracic spinal cord				2		1	
Lumbar spinal cord			1	2		3	

*Number examined.

Table 3

Summary of pathological changes in crab-eating monkeys by flaviviruses.

Inoculum	Control fluid	DEN-2 vaccine	DEN-2 parental	JE-virus	YF-vaccine
Total CNS sites examined (No. of animals × 13)	39	78	39	39	39
No. CNS sites; with					
NV-lesion	2 (5)*	7 (9)	4 (10)	34 (87)	34 (87)
with V-1	2 (5)	7 (9)	4 (10)	20 (51)	30 (77)
with V-2	0	0	0	14 (36)	4 (8)
with NR	3 (8)	26 (33)	12 (31)	5 (13)	3 (10)

*Proportion affected (%)

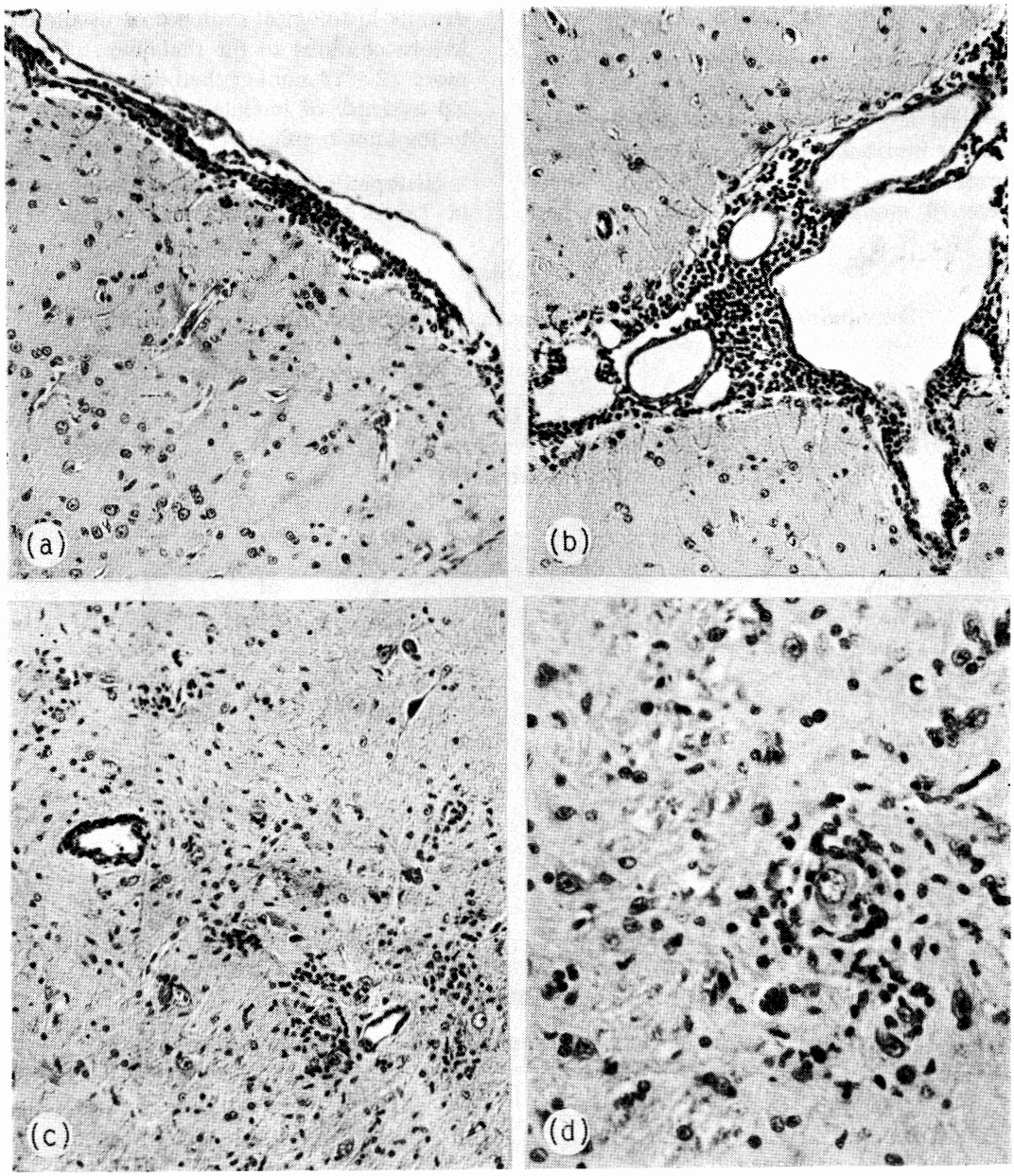

Fig. 1—Crab-eating monkey inoculated with JE-virus. (a)-Frontal cortex showing numerous lymphocytes in leptomeninges (H&E, × 150). (b)-Within the grey matter, infolding of meninges contains cuffs of lymphocytes (H & E, × 150). (c)-Multiple glial nodules with lymphocytic cuffs are seen in the substantia nigra (H & E, × 150). (d)-Glial nodules with and without degenerating neurons are present in the pons (H & E, × 300).

study all developed NV-type lesions in the brain and the spinal cord following inoculation with either JE-virus or YF-vaccine. With both of these viruses, the NV-lesions in the brain were quite disseminated, involving all 4 or the major areas of the cerebral hemispheres examined and the cerebellum. In these areas, there were often significant lymphoid infiltrates (Fig. 1a) and cuffs in the meninges (Fig. 1b) and within Virchow-Robin space, perivascular lymphoid cuffs in the neuropil, glial nodules, and occasional degeneration or necrosis of neurons (Fig. 1c and Fig. 1d). There was a tendency for these lesions to be located in the outer grey matter of the cerebrum and the molecular layer and dentate nucleus of the cerebellum (Fig. 2a). In the remainder of the brain NV-lesions ended to occur in the substantial nigra, putamen, red nucleus, and basal ganglia. In the spinal cord, lesions were located in all 3 major levels examined (cervical, thoracic and lumbar), suggesting that anterior or posterior spread of viral infection had occurred from the lumbar or thalamic inoculation sites, respectively. NV-lesions in the spinal cord associated with inoculation of JE-virus or YF-vaccine virus, regardless of level in the cord, tended to be concentrated in the ventral horns.

In monkeys inoculated with either parental DEN-2 virus or DEN-2 vaccine virus, NV-lesions were identified in only 2 of 3 and 2 of 6 monkeys, respectively. With both viruses, lesions were of minimal (V-1) severity and sparsely scattered, with convincing evidence of neuronal dengeneration or necrosis. Additionally, the few NV-type lesions observed tended to be localized in the same anatomic region as the needle tracts.

DISCUSSION

Neurovirulence studies in rhesus monkeys have been found to be the best presently available laboratory indicator of the potential risk for human inoculated with newly developed poliovirus and group B arbovirus attenuated vaccines (Nathanson *et al.*, 1965;

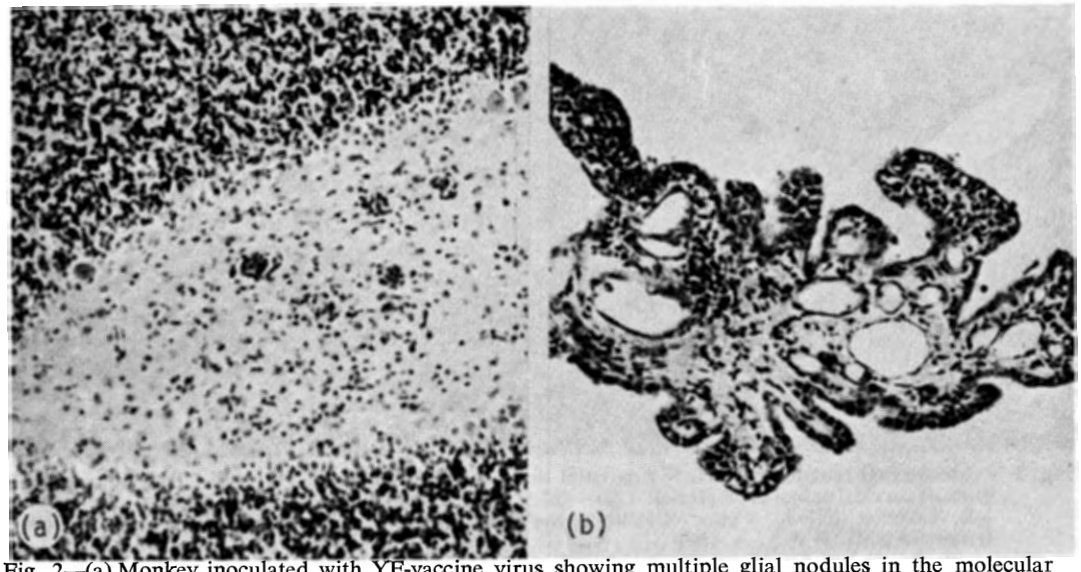

Fig. 2—(a) Monkey inoculated with YF-vaccine virus showing multiple glial nodules in the molecular layer of the cerebellum (H & E, × 150). (b) Monkey inoculated with DEN-2 parental virus showing minimal lymphoid infiltration in the choroid plexus (H & E, × 150).

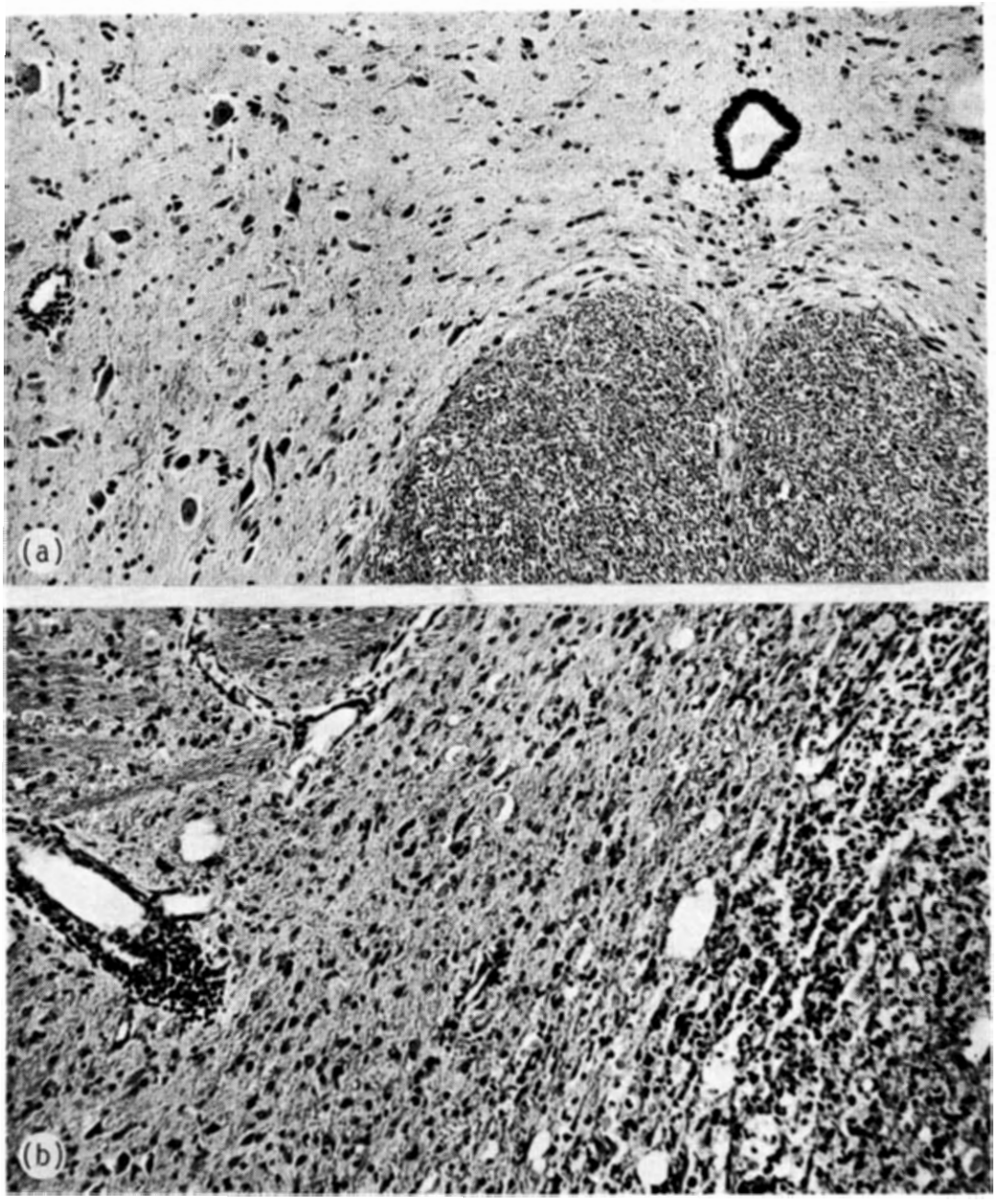

Fig. 3—(a) Monkey inoculated with DEN-2 parental virus showing minimal lymphoid cuffs in the ventral horn of cervical spinal cord (H & E, 150). (b) Monkey inoculated with DEN-2 vaccine virus. The left thalamus showing a zone of minimal malacia and macrophage infiltration with an adjacent lymphoid cuffs (H & E, × 150).

Boulger, 1973). Rhesus monkeys were initially adopted for routine neurovirulence assays of poliovaccine (Murray *et al.*, 1959). Subsequent investigations by using intraspinal (Boulger and Perkins, 1965), intrathalamic (Boulger, 1973), or both intrathalamic and intraspinal inoculations (Boulger *et al.*, 1978) with type I and type III attenuated poliovirus showed that crab-eating monkeys were more sensitive than the rhesus. Our previous studies of the neurovirulence of DEN-2 (Angsubhakorn *et al.*, 1986a), attenuated vaccine virus in the rhesus showed that there was no departure from previously established neurovirulence, and the vaccines have undergone extensive clinical trails in man (Bhamarapravati *et al.*, pers. commun., 1986).

The nature of these lesions was similar to, although apparently more severe than, those seen in our studies (Angsubhakorn *et al.*, 1986a) where rhesus monkeys *(M. mulatta)* were inoculated with parental or attenuated vaccine DEN-2 viruses. Nonspecific response (NR) type lesions were observed more frequently in crab-eating monkeys inoculated with parental or attenuated DEN-2 virus than in those inocuated with DEN-2 control fluids. This greater incidence of NR is not understood. Perhaps it reflects greater antigenicity or toxicity of fluid-cell cultures which have undergone viral infections (cell breakdown products, etc.).

One monkey inoculated with control fluid for DEN-2 had a few lesions in the brain which met the criteria for a V-1 NV-lesion. These lesions consisted of an occasional perivascular lymphoid cuffs in locations remote from the needle track. This observation brings to point the fact that lymphoid cuffs, especially when of minimal severity, do not comprise definitive evidence of neurovirulence. In addition to this report, virus isolation and certain changes between blood chemistry, hematology and serology were observed on pre-and post-inoculation and briefly discussed (Angsubhakorn *et al.*, 1986b).

These studies suggest that the crab-eating monkey is an acceptable host species for neurovirulence testing of dengue vaccines. The crab-eating monkey may, therefore, provide a substitute host species when the more commonly used rhesus monkey is not available. The best method for confirming this proposition would be the parallel, simultaneous testing of each monovalent candidate vaccines in rhesus and crab-eating monkeys.

SUMMARY

The neurovirulent properties of attenuated dengue-2 and yellow fever (YF) vaccines, dengue-2 (DEN-2) and Japanese encephalitis (JE) viruses were studied in crab-eating monkeys *(Macaca fascicularis)*. Number of central nervous system sites (as proportion affected) with neurovirulence (NV) lesions were compared. The results indicate that these monkeys reliably developed NV-lesion when inoculated with either JE or YF vaccine viruses (87%). NV-lesions occurred in a minority when inoculated with DEN-2 vaccine virus, were of minimal severity (9%), were probably biologically insignificant, and were of equal or less severity than lesions produced by its parental virus (10%).

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