

Virological and Pathological Observations
of Dengue Virus Replication in Subcutaneously
Innoculated Rhesus Monkeys

Principal Investigators : Ananda Nisalak, M.D.
 Robert McNair Scott, LTC, MC
 Frank Chapple, MAJ, DVM
 Markpol Tingpalapong, DVM
 Natth Bhamarpravati, M.D.*

Assistant Investigators : Naowayubol Nutkhamhang, B.Sc.
 Nongnard Sahasakmontri, B.Sc.
 Panor Srisongkram, B.Sc.
 Ming Choohong

OBJECTIVE : To examine the skin and the local lymph nodes of experimentally inoculated monkeys for virus isolation and pathological evidence of dengue virus replication.

BACKGROUND : Experimental studies have shown that dengue virus can be recovered from the site of inoculation and from the local draining lymph nodes of Indian Rhesus monkeys (*Macaca mulatta*) (1). Investigations have not been conducted to determine if pathological changes are associated with virus replication. This study was designed to confirm and to extend the virological findings and to examine the local histopathological changes associated with dengue virus replication. In addition, immunofluorescent and electron microscopic studies aimed at identifying the site of dengue virus replication were conducted.

METHODS : The monkeys employed had been used in malaria studies and all had been found to be free of dengue-2 virus neutralizing antibody. Each experimental monkey (F-77, F-78, F-79) was inoculated at nine different sites via the subcutaneous route with dengue-2 virus (BM50-76, LLC-Mk₂-2), 0.5 ml per injection site, (Figure 1). The amount of virus in the inoculum was determined by standard plaque assay at 37°C. employing LLC-Mk₂ cell cultures. One control monkey (F-80) was inoculated with virus-free LLC-Mk₂ tissue culture fluid. Each monkey was anesthetized with phencyclidine hydrochloride and elliptical biopsy specimens were obtained from the skin at 30 minutes, 24, 48, 72 and 96 hours and at days 11 and 15 post-inoculation. Biopsy specimens of local lymph nodes were obtained at 24, 48, 96 hours and on day 7 post-inoculation. Each biopsy specimen was divided into four parts.

One part of each specimen was placed in 4 ml. of Hanks balanced salt (HBS) medium supplemented with 10% calf serum. Specimens were processed for virus isolation according to the explant culture technique (2) of Marchette. Fragments of tissues were washed twice in phosphate buffered saline (PBS) pH 7.9, minced with sterile scissors, and then washed a third time in PBS. The minced tissue was suspended in 4.0 ml of medium 199, 15% calf serum, 1% glutamine and 200 units of penicillin/ml and 200 µg of streptomycin/ml. Each tissue suspension was

* Dept. of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

inoculated onto LLC-Mk₂ cell cultures and incubated at 37°C for 7 days. Medium of cell cultures was removed and replenished every 3 days. Virus isolation attempts were conducted on this medium. On day 7 post-inoculation, cell cultures were subjected to a freeze-thaw cycle and submitted for virus isolation studies. Suspensions of medium and cell cultures were tested for virus by the direct and delayed plaque assay technique.

The remaining portions of each biopsy specimen were sent to the Department of Pathology, Ramathibodi Hospital, Mahidol University for electron microscopy, histopathology and for direct fluorescent antibody studies. One portion was dehydrated at 4°C in a graded series of alcohol and then embedded in epoxy resin. Ultrathin sections were examined with a Hitachi electron microscope, Model HU-12A OY HS-8. Histopathology studies were performed on 10% buffered formalin fixed portions. Specimens for direct F.A. investigation were quick frozen in a dry ice - isopentane mixture. Sections, 4-6 μ thick, were made at -20°C with a cryostat, placed on slides, and air dried. Slides were fixed in 2-octanol at -30°C for 30 minutes, dried at 4°C for one hour and stained with FITC labeled dengue virus antibody.

All monkeys were examined on the day before inoculation and daily throughout the experiment. Blood was obtained from each monkey on day 0 pre-inoculation and on days 1 through 11, and on days 15, 30 and 60 post-inoculation.

Each blood specimen was submitted for hematology studies to determine hemoglobin, hematocrit, white blood count and platelet count. In addition, serum and plasma were tested for antibody, SGOT, SGPT total protein level and for virus, respectively. Virus isolation attempts were performed on peripheral blood leukocytes and on plasma by direct and delayed plaque assay employing LLC-Mk₂ cell cultures at 37°C. Serology was performed by hemagglutination inhibition and complement fixation tests employing Dengue-1-4 and Chikungunya antigens. The plaque reduction neutralization test was performed against dengue-2 virus.

RESULTS : Hematologic and biochemical findings were normal. The results of virus isolation studies performed on monkeys after inoculation of 1.2×10^5 plaque forming units (PFU) of dengue-2 virus per site are presented in Table 1.

Virus was recovered from plasma and skin of all monkeys, whereas leukocytes of only one monkey and lymph nodes of two monkeys yielded virus. The recovery of virus from skin and plasma of the control monkey was probably due to either inadvertent inoculation with a syringe containing virus or contamination of surgical tools used during the performance of biopsies. Lymph nodes taken from this monkey on day 1 and 2, before detectable viremia, served as negative controls for pathology studies. All infected monkeys developed broadly cross-reactive HI antibody to all 4 dengue virus serotypes (Table 2).

Ultrastructural studies of the cells of the primary lymph nodes showed the consistent observation of one or a few viral lattice crystals in the cytoplasm of occasional reticulum cells; in the cytoplasm of the endothelial cells of the capillaries and post-capillary venules, in the cytoplasm of the macrophages, and in the cytoplasm of lymphocytes in the cortical and paracortical areas. Some

plasma cells also were observed to contain the crystals. The lattice crystals measured 0.1 to 3 μ m in size, consisted of individual spherical dense particles of 30-35 nm in a paracrystalline arrangement, and were usually enclosed by a membranous structure associated with the cisterna of the rough endoplasmic reticulum. These viral crystals were not seen in control lymph nodes. Results of histopathology and immunofluorescent studies are not yet complete.

Table 1. Specimens from monkeys yielding dengue-2 virus

Monkey No.	Inoculation	Day Positive Virus Isolation			
		Plasma	Leukocytes	Skin	Lymph node
F-77	BM50-76 Mk ₂ -2 (D-2) 1.1x10 ⁵ PFU SQ x 9	1,4,5,6	-	4,5	4,7
F-78	BM50-76 Mk ₂ -2 (D-2) 1.1x10 ⁵ PFU SQ x 9	2,4	5	1,2,7	2,7
F-79	BM50-76 Mk ₂ -2 (D-2) 1.1x10 ⁵ PFU SQ x 9	2,4,5	-	3	-
F-80	Uninfected Mk ₂ culture fluid SQ x 9	3,4,5,6	-	5,7	-

Table 2. Reciprocal hemagglutination inhibition titers by day following inoculation of monkeys with dengue-2 virus

Monkey Number	Day Following Inoculation*	Reciprocal Hemagglutination Inhibition Titers				
		Den-1	Den-2	Den-3	Den-4	CHICK
F-77	D-0	<10	<10	<10	<10	<10
	D-15	320	1280	1280	2560	<10
	D-30	80	320	160	640	<10
	D-60	40	160	80	320	<10
	D-180	80	320	160	320	<10
F-78	D-0	<10	<10	<10	<10	<10
	D-15	160	640	640	2560	<10
	D-30	80	160	320	640	<10
	D-60	40	160	80	320	<10
	D-180	40	80	80	320	<10
F-79	D-0	<10	<10	<10	<10	<10
	D-15	160	640	320	1280	<10
	D-30	80	320	160	640	<10
	D-60	80	160	80	320	<10
	D-180	80	160	160	320	<10
F-80	D-0	<10	<10	<10	<10	<10
	D-15	80	320	160	640	<10
	D-30	80	160	160	320	<10
	D-60	40	160	40	320	<10
	D-180	20	80	40	40	<10

* Inoculum BM50-76 (LLC-Mk₂-2) 1.1×10^5 PFU.

REFERENCES :

1. Marchette, N.J., Halstead, S.B., Falkler, W.A., *et al.*, 1973. Studies on the Pathogenesis of Dengue Infection in Monkeys. III. Sequential Distribution of Virus in Primary and Heterologous Infections. *J. Inf. Dis.* 128:23-30.
2. Marchette, N.J., Halstead, S.B., Nash, D.R., *et al.*, 1972. Recovery of Dengue Viruses from Tissues of Experimentally Infected Rhesus Monkeys. *Applied Microbiol.* 24:328-33.

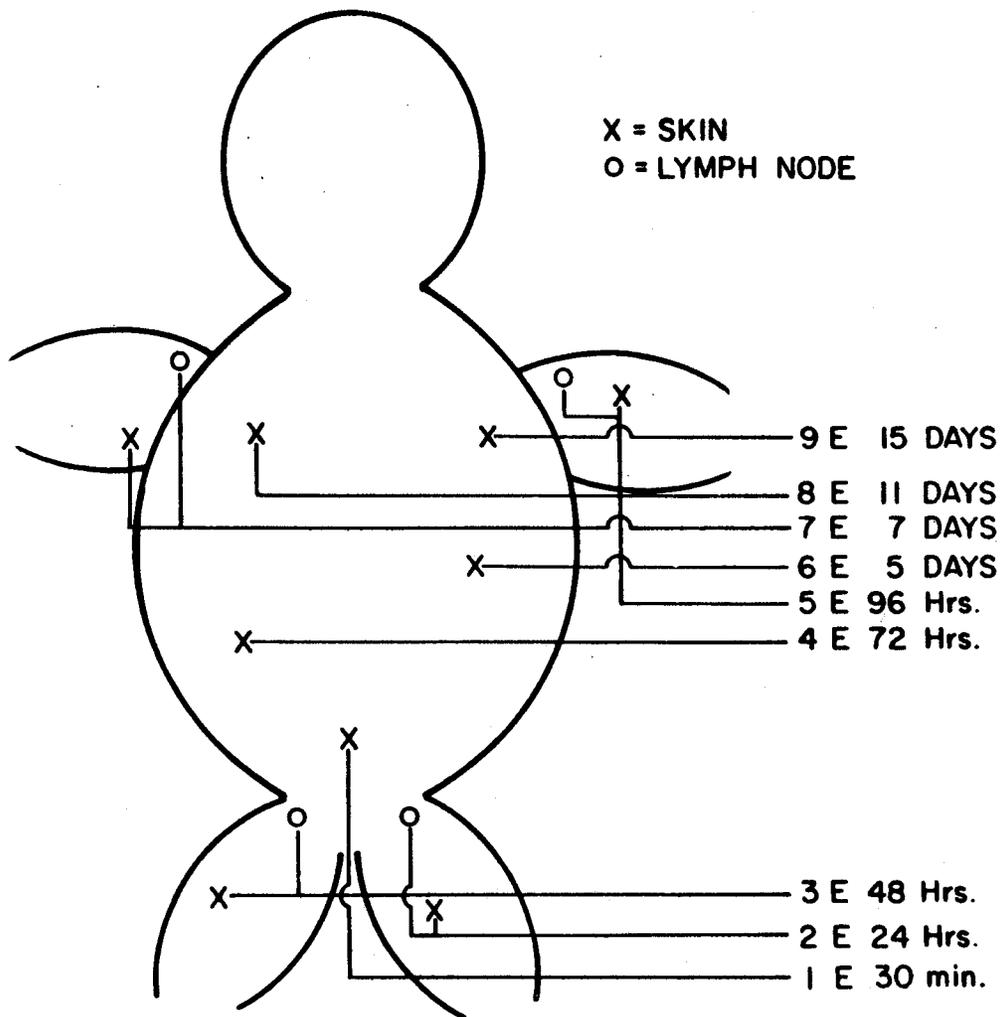


FIGURE I. SKIN AND LYMPH NODE BIOPSY SITES