

Title: Arbovirus Infection in Man and Experimental Animals

Principal Investigators: Ananda Nisalak, M.D.
Pairatana Gunakasem, M.D.
Philip K. Russell, LTC MC
Phinit Simasathien, M.D.
Suchinda Udomsakdi, M.D.
Robert H. Whitehead, M.Sc.
Thomas M. Yuill, Ph.D.

Associate Investigators: Anong Intavivat, M.Sc.
Pisamai Janravavijuksu, M.Sc.
Sopana Kanchanapilant, M.D.

Assistant Investigators: Chinda Charupat, M.Sc.
Malee Chuengcharoen, M.Sc.

Objectives

To develop improved virologic and serologic methods for diagnosis and epidemiologic study of arbovirus diseases in Southeast Asia.

To determine the antigenic relationships and biologic properties of arboviruses of medical importance.

To investigate in detail the immunologic response of man and experimental animals to single and multiple dengue infections and to relate such observations to disease processes.

Description

Dengue virus strains isolated in the course of the epidemiologic studies described above were identified by plaque reduction neutralization tests (PRNT). Various factors which affect PRNT results were investigated and an attempt was made to determine the extent of antigenic variation within dengue serotypes.

A microplaque method for measuring neutralizing antibody to dengue, Japanese encephalitis and Wesselsbron viruses was developed and standardized for use in epidemiologic investigations.

Immunochemical methods were used to determine the types of antidengue immunoglobulins produced in the course of dengue infections in children with dengue shock syndrome. Serial measurements of Beta_{1c/a} globulins levels in serum were made in shock syndrome patients.

Cross protection between dengue serotypes is being investigated by subcutaneous inoculation of gibbons (*Hylobates lar*) using serologic response and viremia as parameters for determining the extent of cross protection conferred by infection.

Results

Identification of Dengue Viruses

The epidemiologic studies reported above and other investigations of dengue hemorrhagic fever cases required identification of more than 90 strains of dengue viruses. These viruses were identified by plaque reduction neutralization tests (PRNT) with monkey antiserum using methods described in the previous annual

report. In the course of completing the identification of this large number of agents several problems became apparent. The major difficulty was the relative difficulty with which some low passage tissue culture seed viruses were neutralized by immune sera. This low "neutralizability" was reported previously for several dengue 2 strains. A similar phenomenon has also been observed with some viruses in the other dengue serotypes. Attempts to explain this phenomenon of low neutralizability have been only partially successful (see below), and because of the relative difficulty with neutralization of some low passage viruses strains it was necessary to use neutralizing antiserum with relatively high titers and high specificity. Antisera made to Asian strains of dengue virus in low passage have proven to be superior in this regard than antiserum against prototype strains. This was most apparent with dengue-4 antiserum. Several attempts to make monkey antiserum to the prototype H-241 strain of dengue-4 resulted in sera with low PRNT titers. The 24038 strain (Vietnam 1966) of dengue-4 produced highly specific antiserum with an acceptable titer. The PRNT titers versus homologous and prototype viruses of the antisera finally selected for use in identification tests are given in table 7.

Table 7. Neutralizing antibody titers of antisera used for virus identification vs. homologous and prototype dengue viruses.

Virus Strains	Antisera			
	Dengue-1 (12900)	Dengue-2 (BKM-551)	Dengue-3 (Pak-18)	Dengue-4 (24038)
Dengue-1 (Hawaii)	180 ^{1/}	30	15	0
Dengue-1 (12900)	320			
Dengue-2 (N.G. "C")	10	640	0	0
Dengue-2 (BKM-551)	0	> 640		
Dengue-3 (H-87)	0	0	350	0
Dengue-3 (Pak-18)			350	
Dengue-4 (H-241)	0	0	0	90
Dengue-4 (24038)				170

^{1/} Reciprocal of 50% plaque reduction titer, 0 = <10

The 39 dengue viruses recovered from mosquitoes and patients on Koh Samui all reacted similarly with the reference antisera. Results of identification for six representative strains are given in table 8. Reactions were seen only with the dengue-4 antisera. Some degree of variation in titer of the reference antiserum to the individual strains is apparent. However, differences are of questionable significance and may be due to factors other than differences in antigenic composition. All of these strains produced uniform, relatively large (2-3 mm) plaques in LLC-MK₂ cell culture.

Table 8. Neutralizing antibody titers of reference dengue antisera vs. dengue strains from Koh Samui 1967.

Strain No.	Source	Passage ^{1/} Level	Antisera			
			D-1	D-3	D-4	
KS-52	<u>A. aegypti</u>	1	0 ^{2/}	0	0	40
KS-168	"		0	0	0	170
KS-197	<u>A. albopictus</u>	1	0	0	0	40
KS-273	"	2	0	0	0	200
29675	patient	2	0	0	0	250
30401	"	2	0	0	0	35
(homologous viruses)			(320)	(> 640)	(350)	(170)

^{1/} Passage level in LLC-MK₂ cell culture.

^{2/} Reciprocal of 50% plaque reduction titer, 0 = <10

Dengue viruses recovered from Aedes aegypti collected in Saigon were identified using the same antisera. The results of identification tests for representative strains of each serotype are given in table 9. Additional neutralization tests using antisera to prototype strains as well as the reference antiserum were carried out with several dengue-1 and dengue-2 strains from Saigon. Results of these tests shown in table 10 indicate a consistent difference between the recent isolates and the prototype strains. This appears to be independent of "neutralizability" and probably is due to an antigenic difference.

Table 9. Neutralizing antibody titers of reference dengue antisera vs. dengue strains from Saigon 1967.

Virus Strain	Passage Level ^{1/}	Antisera			
		D-1	D-2	D-3	D-4
BKM-704	2	300 ^{2/}	0	0	0
BKM-725	2	500	0	0	0
BKM-773	2	0	640	0	0
BKM-1311	2	0	170	0	0
BKM-1552	4	0	> 640	0	0
BKM-987	2	0	30	> 640	40
BKM-1547	1	0	0	0	40
BKM-1823	2	0	0	20	320
(homologous viruses)		(320)	(> 640)	(350)	(170)

^{1/} Passage level in LLC-MK₂ cell culture.

^{2/} Reciprocal of 50% plaque reduction titer, 0 = <10

Table 10. Comparison of dengue-1 and dengue-2 strains from Saigon with prototype strains by neutralization tests.

Virus Strain	Antisera			
	Dengue-1 (Hawaii)	Dengue-1 (12900)	Dengue-2 N.G. "C"	Denge-2 BKM-551
Dengue-1 (Hawaii)	320*	250	0	0
Dengue-1 (12900)	50	320	0	0
BKM-704	80	300	0	0
BKM-725	90	500	0	0
BKM-1197	80	640	0	0
Dengue-2 (N.G. "C")	0	0	640	640
Dengue-2 (BKM-551)	0	0	160	> 640
BKM-773	0	0	45	640
BKM-1311	0	0	40	170
BKM-1552	0	0	37	> 640

* reciprocal of 50% plaque reduction titer, 0 = <10

Strains of dengue viruses recovered during the course of investigating dengue outbreaks in Malaysia were submitted to this laboratory by Dr. Albert Rudnick of the Hooper Foundation Arbovirus Research Laboratory, University of Malaya, Kuala Lumpur. Seventeen strains have been identified. One strain was recovered from Aedes aegypti and the remainder from human patients. Four geographical areas are represented. All strains were isolated in suckling mice. The origin of the strains and the serotype are given in table 11. The neutralization test data by which these strains were identified is given in table 12. Three serotypes are represented.

Table 11. Source, passage level and serotype of 1967 Malaysian dengue viruses^{1/}

Strain No.	Date		Locality	Serotype
	Collected			
P7-337	31 Mar 67		Petaling Jaya	dengue-4
P7-570	8 June 67		" "	dengue-4
P7-601	10 July 67		" "	dengue-2
P7-612	13 July 67		Kuala Lumpur	dengue-4
P7-673	15 July 67		Ipoh	dengue-4
P7-686	24 July 67		Petaling Jaya	dengue-2
P7-772	29 June 67		" "	dengue-4
P7-776	15 Aug 67		Penang	dengue-4
P7-845	10 July 67		Petaling Jaya	dengue-2
P7-852	28 Aug 67		Penang	dengue-4
P7-931	15 Sept 67		"	dengue-4
P7-974	10 Sept 67		Petaling Jaya	dengue-4
P7-1006 ^{2/}	13 Sept 67		Kuala Lumpur	dengue-4
P7-1019	28 Sept 67		Penang	dengue-4
P7-1024	7 Oct 67		"	dengue-3
P7-1029	29 Sept 67		"	dengue-4
P7-1032	7 Oct 67		"	dengue-4

^{1/} Viruses recovered by Dr. Albert Rudnick, Hooper Foundation
Arbovirus Research Laboratory, University of Malaya, Kuala Lumpur.

^{2/} From A. aegypti; remaining strains from human sera.

Table 12. Neutralizing antibody titers of reference monkey antisera vs. Malaysian dengue viruses

Strain No.	Antisera			
	D-1	D-2	D-3	D-4
P7-337	0*			60
P7-570	0			250
P7-601	0			0
P7-612	0			60
P7-676	0			50
P7-686	0			20
P7-772	0			230
P7-776	0			120
P7-845	0			0
P7-852	0			100
P7-931	0			50
P7-974	0			80
P7-1006	0			190
P7-1019	0			40
P7-1024	0			10
P7-1029	0			120
P7-1032	0			50
(homologous viruses) (320)				(170)

* Reciprocal of 50% plaque reduction titer, 0 = <10

A strain of dengue-3 (502066) recovered from a patient with dengue in Tahiti in 1965 by Dr. Leon Rosen, Pacific Research Section, NIAID was compared with other dengue-3 strains. Antisera made in monkeys by a single subcutaneous injection of live virus were used. Results of neutralization tests, given in table 13, indicate a difference between the Tahiti 502066 strain and the other strains tested. Although antisera to the Asian strains neutralized the Tahiti virus at titers comparable to homologous titers, antisera made of the Tahiti virus had titers more than 10-fold lower than the homologous titer when tested against the prototype H-87 and three other dengue-3 strains. This difference, though seen only one way, appears to indicate an antigenic variations.

Table 13. Comparison of dengue-3 viruses by neutralization tests.

<u>Virus Strains</u>	<u>Pak-18</u>	<u>Monkey Antisera</u>		<u>502066</u>
		<u>21153</u>	<u>24969</u>	
H-87 (Manila 1956)	320 ^{1/}	25	120	13
Pak-18 (Dacca 1964)	<u>300</u>	32	160	20
21153 (Bangkok 1965)	<u>300</u>	<u>30</u>	160	10
24969 (Koh Samui 1966)	> 640	160	<u>130</u>	60
502066 (Tahiti 1965) ^{2/}	180	70	150	<u>640</u>

^{1/} Reciprocal of 50% plaque reduction titer.

^{2/} Virus isolated by Dr. Leon Rosen, Pacific Research Section, NIAID, Honolulu.

Plaque Reduction Neutralization Tests

Several low passage tissue culture strains of dengue-2 viruses have been observed to be poorly neutralized by antisera while higher passages and mouse passage lines of the same strain are more readily neutralized by the same antisera. The studies on dengue-2 (BKM-551) virus reported previously exemplify this phenomenon. Possible explanations for this phenomena include aggregation of viruses or viruses and tissue culture products, presence of non-infectious viral antigen which binds to antibody, difficulties in plaque count assays due to plaque size variants, antigenic differences in the virus, or substances in tissue culture seed viruses which interfere with the neutralization reaction.

To determine if aggregation of virus particles was occurring in tissue culture seed preparations, three dengue-2 seed virus preparations were subjected to ultrasonication at 10,000 cps for 5 minutes. Infectivity titers of control and ultrasound treated virus seeds were unchanged (table 14). Neutralization tests before and after ultrasonication with the BKM-551 p6 virus gave similar results. Further, centrifugation at 12,000 rpm for 30 minutes and filtration through 450 mu filters did not affect the infectivity titer of a BKM-551 seed virus preparation. Therefore it appears unlikely that virus aggregation is a significant factor in affecting the results of plaque reduction neutralization tests.

Table 14. Effect of ultrasonication on dengue-2 virus seeds produced in LLC-MK₂ cell culture.

<u>Virus</u>	<u>Passage</u>	<u>Infectivity (pfu/0.3 ml)</u>	
		<u>Control</u>	<u>Ultrasonicated</u>
BKM-551	6		3×10 ⁵
29681	2		4×10 ³
KS-197	1		2.5×10 ³

Plaque Variants of Dengue-2 (BKM-551) Virus

Several strains of dengue-2 viruses isolated from mosquitoes and patients sera in LLC-MK₂ cell culture have been observed to produce mixtures of large and small plaques under agar. The BKM-551 strain of dengue-2 recovered from mosquitoes from Koh Samui in 1966 was selected for investigation of this phenomena because it has been well characterized and prominent plaque size variation has been observed at several passage levels. The strain under investigation had been isolated and serially passaged in LLC-MK₂ cell culture. Ninth passage seed virus was used for cloning experiments. Antiserum used in these experiments was prepared in a monkey by a single subcutaneous injection of approximately 1,000 pfu of fourth passage virus. The first clones were obtained by performing a plaque reduction neutralization test with homologous antiserum and selecting plaque bottles in which only a few plaques of a single size appeared. Single plaques were picked and when reinoculated on LLC-MK₂ monolayers and overlaid produced plaques of uniform size. Figure 8 depicts the variation in plaque size seen with the parent (p-9) seed virus. The large plaques measure 3 to 4 mm. and the small plaques less than 1 mm. Figures 9 and 10 show the clones derived (p-10) by picking single plaques, the large plaques measure > 2.5 mm and the small plaques approximately 1 mm. Plaque sizes for each clone remained constant after two additional passage under agar. Tenth passage virus of each clone was inoculated into fluid cultures and virus harvested after 8 days incubation. Infectivity titers of this p 11 seed were 4×10^6 pfu/0.3 ml for the small plaque clone and 5×10^5 pfu/0.3 ml for the large plaque clone. Plaque reduction neutralization titers of the BKM-551 antiserum were 1:160 versus the small plaque variant and 1:180 versus the large plaque variant. Using the same serum diluted 1:5, log neutralization indices were greater than 4.5 for each clone. Additional experiments are in progress to determine the stability of these clones and their antigenic and biologic characteristics.

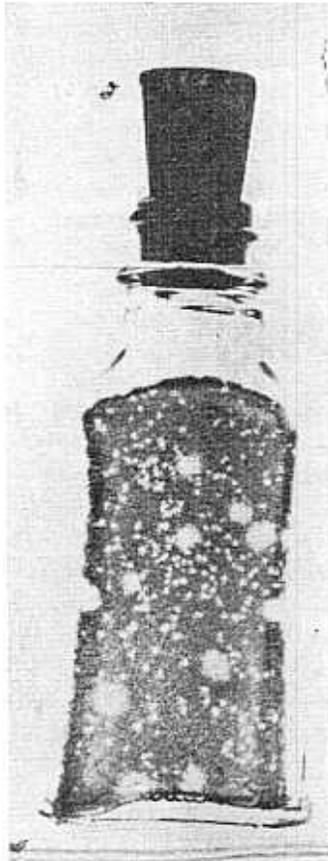


Figure 8. Plaque size variants of dengue-2 (BKM-551 9th LLC-MK₂ passage).

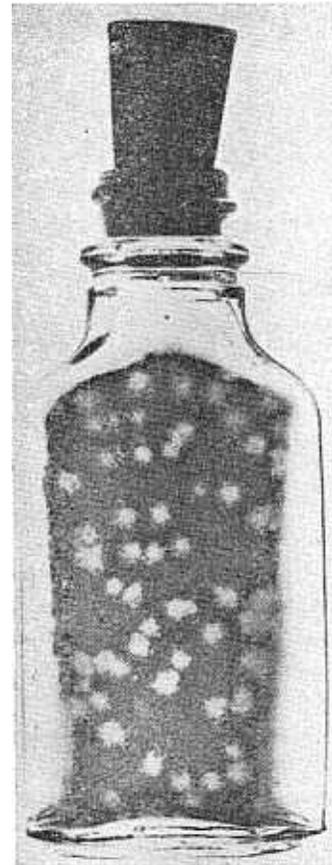


Figure 9. Clone of large plaque variant (BKM-551 10th LLC-MK₂ passage).



Figure 10. Clone of small plaque variant (BKM-551 10th LLC-MK₂ passage).

Development of Micro Methods for Neutralization Tests

Work has continued on development of simple and efficient neutralization tests for use in routine serological studies. The BHK-21 cell metabolic inhibition (MI) test has been further evaluated and is now routinely used for detection of Japanese encephalitis (JE), chikungunya (chik), Sindbis, and Wesselsbron (Wess) virus antibody in animal and human sera. To meet requirements for efficient neutralization tests for viruses, such as dengue, which do not produce cytopathic effect, or when greater precision than is afforded by the MI test is desired, another neutralization method, the microculture plaque reduction neutralization test, has been developed.

The specificity of neutralization of arboviruses known to be in Thailand, in the MI test, has been studied. Very little heterologous cross neutralization within antigenic groups was observed. Antisera to the two Group A viruses, chikungunya and Sindbis, did not cross neutralize at all, even when tested with low (20-30 TCID₅₀) doses of virus (table 15). Similarly, there was relatively little neutralization of JE virus by other Group B antisera. Tembusu and dengue-2 antisera did not neutralize JE virus and antisera to dengue 1 and 4 viruses neutralized JEV at low titer (table 16).

Table 15. Reciprocal metabolic inhibition neutralization tests with Chikungunya and Sindbis viruses and antisera.

Virus (Strain)	Dose ^{1/}	Antisera	
		Chik	Sindbis
Chikungunya (25263)	20	100 ^{2/}	<3
Sindbis (Cglt 599)	30	<10	<u>48</u>

^{1/} Virus dose (TCID₅₀) in the MI test.

^{2/} Reciprocal of serum dilution providing 50% protection of cells.

Table 16. Homologous and heterologous metabolic inhibition (MI) neutralization tests of Japanese encephalitis virus (JEV) by antisera to certain other group B viruses.

Antisera	MI Titer	Homologous
	to JEV ^{1/}	PRNT Titer ^{2/}
Japanese encephalitis	80	3,500
Tembusu	<10	18,000
Dengue-1	15	320
Dengue-2	<10	400
Dengue-3	<10	320
Dengue-4	15	90

^{1/} Reciprocal of serum dilution protecting 50% of the BHK-21 cells against 30-50 LD₅₀ in metabolic inhibition (MI) test.

^{2/} Reciprocal of serum dilution neutralizing 50% of 30-50 plaque-forming units in LLC-MK₂ cell bottle plaque reduction neutralization tests.

The microculture PRNT technique is fairly simple. LLC-MK₂ cells were grown in sterile plastic plates with wells having a bottom diameter of 1.5 cm. To each well 70,000 live cells in 0.7 ml of growth medium were added. Glycerine was applied around the edges of the plate before wrapping with Saran Wrap. The wrapped plates were incubated at 37°C until monolayers formed (usually 4 days). On the day of the test, serum dilutions were made in tubes and 0.1 ml of each dilution was transferred to a well of a disposable microtiter plates. An equal volume of virus calculated to contain 15-20 plaque-forming units (PFU) per 0.025 ml was added. A titration of the virus test dose was made and added to equal volumes of diluent. Glycerine was added to the edges of the microtiter plate to insure a tight seal of the Saran Wrap and the wrapped plate incubated at 37°C for 1 hour. During incubation of serum-virus mixtures, growth medium was removed from the cell sheets by aspiration. The cells were washed with 0.5 ml of Hanks balanced salt solution pH 8.3. To each well 0.025 ml each of diluent and of serum-virus mixture was added. This volume was adequate to avoid drying of cells during the 37°C 2 hour adsorption period. Frequent agitation insured uniform dispersal of virus over the cell sheet. At the end of the adsorption period 0.3 ml of first agar overlay medium was added. The plates were tightly rewrapped and incubated at 37°C for 3 (for JE virus) or 6 (for dengue and West viruses) days when the second agar overlay containing neutral red was added. Plaques were counted the following day.

The microculture PRNT is considerably less cumbersome than similar tests in 1 oz bottles and permits testing of about 3 fold more specimens in a given period of time. The microculture PRNT is not without its limitations however, since the surface area of the cell monolayer is 5 1/2 times less than 1 oz bottle

cultures, the acceptable range in plaque numbers per well becomes much more narrow. If too few plaques (a mean of less than 5 per well) are used, neutralization may be obscured by random variation in plaque numbers. If plaque numbers exceed 15 per well, plaque overlapping results in underestimation of PFU and a consequent underestimation of serum 50% end points. When the virus dose per well is within an acceptable range, the microculture PRNT is reproducible. In three independent assays of dengue 2 virus antisera made by two individuals, neutralization titers over a virus dose range of 5–15 PFU per well gave essentially similar results (table 17).

Table 17. Reproducibility of homologous dengue—2 plaque reduction neutralization tests conducted by two workers on two different occasions.

Test	Worker	Virus	Serum
<u>Date</u>		<u>Dose^{1/}</u>	<u>Titer^{2/}</u>
6 Nov	P	11	225
		6	320
	S	15	310
26 Dec	P	14	250
	S	9	340
		5	270

^{1/} Average number of pfu/well.

^{2/} Reciprocal of 50% plaque reduction titer.

Titers and specificity of homologous and heterologous neutralization by antisera to dengue 1–4 and JE viruses in micro and bottle culture PRNT were the same (table 18). As with bottle culture PRNT, the microculture system is considerably more specific than the hemagglutination–inhibition (HI) test while its greater efficiency makes it a more useful device than the bottle PRNT in establishing serologic diagnosis in cases where HI reactions are complicated by group cross reactions. For example, it was possible to exclude JE virus as an etiologic agent by microculture PRNT antibody assay of acute and convalescent phase sera from two patients with febrile illnesses and establish a serological diagnosis of dengue virus infection (table 19). Although less precise than the bottle culture PRNT, the microculture PRNT appears to be quite satisfactory as a serological tool to (1) detect arbovirus antibodies and (2) measure differences in quantities of arboviral antibodies of the order of magnitude associated with infection. It has the added advantage of requiring smaller volumes of reagents and test sera.

Immunologic Response to Dengue Infection in Man

Epidemiologic, serologic and clinical studies of dengue hemorrhagic fever have provided evidence that the severe form of the disease known as the dengue shock syndrome is associated in the secondary type antibody response to dengue viruses. An immunologic mechanism is suspected of being involved in the pathogenesis of the vascular permeability which is responsible for the shock phase. Ultimate proof of this "second infection" hypothesis will depend on demonstration of the pathogenic mechanism. The immunologic mechanisms in patients with dengue shock syndrome have been under investigation in this laboratory during the past three years. Results reported in previous annual reports have indicated that patients with dengue shock syndrome have an antibody response characterized by a rise in hemagglutination–inhibiting Ig–G antibody. Preliminary results of a study utilizing DEAE–cellulose chromatography to separate immunoglobulins for measurement of anti–dengue antibody activity have been reported. The present report includes data on additional patients with dengue shock syndrome and studies of neutralizing and complement fixing activity of immunoglobulin fractions.

Table 18. Neutralizing antibody titers of sera tested in LLC-MK₂ cell microculture and in one ounce bottle culture plaque reduction neutralization tests.

Virus	Dengue-1		Dengue-2		Dengue-3		Dengue-4		JE (Nak)	
	Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro
Dengue-1	400 ^{1/}	440	10	34	<10	<10	<10	<10	40	ND
Dengue-2	<10	<10	400	> 640	<10	<10	<10	10	10	ND
Dengue-3	<10	<10	20	10	200	270	<10	<10	10	ND
Dengue-4	<10	<10	20	<10	<10	<10	80	90	<10	ND
JE	<10	<10	<10	<10	<10	<10	<10	<10	1280	2560

^{1/} Calculated dilution at which 50% plaque reduction occurs.

27

Table 19. Relative specificity of the hemagglutination-inhibition test vs. the microplaque reduction neutralization test (MPRNT) with serum pairs from two dengue fever patients.

Serum	HI Titer of Antigens					MPRNT Titer to Viruses				
	Dengue-1	Dengue-2	Dengue-3	Dengue-4	JE ^{1/}	Dengue-1	Dengue-2	Dengue-3	Dengue-4	JE
Acute	80 ^{2/}	80	320	160	160	160 ^{3/}	160	40	20	<10
Conval.	640	640	2160	1280	1280	160	> 2560	160	40	<10
Acute	<20	<20	<20	<20	<20	10	<10	<10	10	<10
Conval.	160	320	320	640	160	160	1000	100	10	<10

^{1/} Japanese encephalitis virus.

^{2/} Reciprocal of serum dilution inhibiting 8 units of hemagglutinin. Serum acetone extracted prior to testing.

^{3/} Antibody titer is the reciprocal the highest dilution of serum producing 50 percent plaque reduction.

Patients admitted to Children's Hospital, Bangkok were selected for study on a basis of typical clinical manifestations of dengue hemorrhagic fever with shock as manifested by narrowed pulse pressure, a drop in systolic blood pressure below 90 mm Hg and an elevated hematocrit. Sera were obtained daily during the acute phase and one or more convalescent sera were obtained. The methods of antibody fractionation by DEAE-cellulose chromatography methods for immunoglobulin assay were as described previously.

Table 20 summarizes the HI antibody response of 12 patients studied. In all cases, high titers were present during the shock phase of the illness. The result of fractionations by DEAE cellulose chromatography of the serum pools from these patients is given in table 21 with the dengue HI antibody activity of the various fractions. Fractions I in each case contains only Ig-G, fraction II and III contain Ig-A and Ig-M respectively with small residual amounts of Ig-G.

Table 20. Dengue HI titers of sera and serum pools from patients with dengue shock syndrome.

Case No.	Day Serum Obtained ^{1/}	HI Antibody Titer			
		D-1	D-2	D-3	D-4
HFI-773	<u>6</u> ^{2/}	320	640	2560	2560
	<u>7</u> ^{2/}	640	2560	> 20480	2560
	8	2560	2560	> 20480	> 20480
	9	2560	2560	> 20480	> 20480
	pool	1280	1280	> 20480	> 20480
HFI-747	<u>3</u> ^{2/}	5120	10240	> 20480	> 20480
	7	> 20480	> 20480	> 20480	> 20480
	9	> 20480	> 20480	> 20480	> 20480
	13	> 20480	> 20480	> 20480	> 20480
	pool	> 20480	> 20480	> 20480	> 20480
HFI-749	<u>5</u> ^{2/}	> 20480	> 20480	> 20480	> 20480
	10	> 20480	> 20480	> 20480	> 20480
	20	> 20480	> 20480	> 20480	> 20480
	30	1280	<u>1280</u>	1280	5120
	pool	10240	10240	10240	20480
HFI-744	<u>5</u> ^{2/}	640	640	5120	640
	6	5120	5120	20480	2560
	8	2560	1280	20480	1280
	20	1280	1280	20480	2560
	pool	5120	1280	20480	2560
HFI-737	<u>5</u> ^{2/}	320	640	1280	640
	10	> 20480	> 20480	> 20480	> 20480
	20	10240	10240	20480	5120
	30	10240	10240	> 20480	5120
	pool	> 20480	> 20480	> 20480	5120
HFI-782	<u>4</u> ^{2/}	5120	1280	5120	2560
	5	10240	1280	10240	10240
	6	> 20480	5120	> 20480	> 20480
	pool	> 20480	5120	10240	10240

^{1/} Day after onset of illness.

^{2/} Days on which shock was observed.

Table 20. (Continued)

Case No.	Day Serum Obtained ^{1/}	HI Antibody Titer			
		D-1	D-2	D-3	D-4
HFI-761	3 ^{2/}	640	640	640	640
	4 ^{2/}	1280	1280	5120	2560
	5	2560	10240	10240	5120
	pool	2560	2560	2560	2560
HFI-764	10 ^{2/}	1280	1280	640	640
	11 ^{2/}	2560	2560	1280	2560
	12 ^{2/}	2560	2560	2560	2560
	pool	2560	2560	2560	5120
HFI-770	4 ^{2/}	2560	2560	5120	1280
	12	20480	10240	10240	10240
	35	10240	2560	5120	2560
	pool	5120	2560	10240	2560
HFI-789	5 ^{2/}	2560	320	2560	1280
	6	5120	2560	5120	2560
	7	2560	5120	5120	5120
	pool	640	1280	2560	2560
HFI-794	5 ^{2/}	160	640	80	80
	6	320	640	640	160
	7	640	1280	1280	640
	pool	640	1280	1280	640
HFI-795	4	20	40	20	80
	5 ^{2/}	160	80	320	160
	6 ^{2/}	640	640	640	640
	pool	640	640	640	640

^{1/} Day after onset of illness.

^{2/} Days on which shock was observed.

Complement fixing antibody activity was measured in all three fractions from each serum pool. In every case activity was found only in fraction I and in each case high titers and broad cross-reactivity were seen (table 22). The neutralizing antibody activity in each fraction was measured by plaque reduction neutralization tests and results, summarized in table 23, are comparable to the HI tests. Most N antibody activity was found in fraction I associated with Ig-G. The high and broadly cross-reactive neutralizing antibody titers are characteristics of second dengue infections.

The results presented above indicated that in patients with dengue shock syndrome, the HI, CF, and neutralizing antibody activity to dengue viruses was associated with Ig-G. Contamination of fraction II with small amounts of residual Ig-G precludes making a firm conclusion that no anti-dengue Ig-A activity was present in these serum pools, however, the amount of contaminating Ig-G appeared sufficient to account for the low level for activity seen in fractions II and III. Further if anti-dengue Ig-A antibody activity was present, it was extremely small amounts. Since all HI antibody activity in fraction III was found to be resistant to 2-mercaptoethanol, the antidengue activity of Ig-M was also negligible in these cases.

Table 21. Immunoglobulin concentrations and HI antibody titers of fractions from DEAE-cellulose chromatography of serum pools from patients with dengue shock syndrome.

Case No.	Fraction No.	Immunoglobulin Conc.			HI Titer ^{1/}
		Ig-G	Ig-A	Ig-M	
HFI-773		560 ^{2/}	0	0	2560
	II	15	20	0	40
	III	40	0	98	160
HFI-747		440	0	0	10240
	II	30	66	0	160
	III	30	tr. ^{3/}	86	160
HFI-749		520	0	0	10240
	II	20	70	0	80
	III	20	tr.	110	80
HFI-744		280	0	0	10240
		tr.	30	0	40
	III	tr.	0	46	20
HFI-737		450	0	0	10240
	II	tr.	32	0	20
	III	tr.	0	30	20
HFI-782		280	0	0	5120
	II	tr.	31	0	80
	III	tr.	0	15	40
HFI-761		350 ^{2/}	0	0	640
	II	tr. ^{3/}	35	0	0
	III	tr.	0	15	0
HFI-764		350	0	0	1280
	II	15	50	0	40
	III	10	0	16	20
HFI-770		350	0	0	5120
	II	15	35	0	40
	III	10	0	15	20
HFI-789		350	0	0	640
	II	12	40	0	80
	III	12	0	15	20
HFI-794		480	18	0	1280
	II	19	35	0	20
	III	17	0	20	20
HFI-795		508	23	0	640
	II	20	51	0	0
	III	12	0		0

^{1/} Reciprocal of titer vs. 8 units of the dengue antigen which gave highest titer.

^{2/} mg. %.

^{3/} Trace, <10 mg. %.

Table 22. Dengue CF antibody titers in fraction I (Ig-G) from DEAE-cellulose chromatography of serum pools from patients with dengue shock syndrome^{1/}.

Case No.	CF Antibody Titer ^{2/}			
	D-1	D-2	D-3	D-4
HFI-773	32	32	64	128
HFI-747	32	256	32	128
HFI-749	16	64	16	32
HFI-744	64	256	8	64
HFI-737	32	32	32	16
HFI-782	128	256	128	256
HFI-761	32	16	64	128
HFI-764	32	16	64	64
HFI-770	64	16	128	64
HFI-789	16	128	128	64
HFI-794	32	64	64	16
HFI-795	4	8	128	32

^{1/} In each case no CF antibody was detectable in fractions II or III at 1: 2 dilution.

^{2/} Reciprocal of titer vs. 4 units of indicated antigen.

Table 23. Dengue neutralizing antibody titers of fractions from DEAE-cellulose chromatography of serum pools from patients with dengue shock syndrome.

Case No.	Fraction No.	Antibody Titer ^{1/}			
		D-1	D-2	D-3	D-4
773	I	640	640	> 2560	<10
	II	<10	<10	40	<10
	III	40	10	80	<10
	I	640	640	> 2560	40
	II	160	40	10	10
	III	80	40	20	<10
	I	> 2560	2560	640	40
	II	160	40	40	<10
	III	40	80	40	<10
749	I	> 2560	2560	> 2560	2560
	II	20	<10	160	10
	III	> 160	160	160	20
	I	> 2560	1000	2560	2560
	II	160	<10	160	100
	III	20	<10	100	<10
	I	> 2560	2560	2560	> 2560
	II	40	<10	20	10
	III	<10	<10	<10	<10
761	I	1280	640	1280	640
	II	10	<10	20	<10
	III	<10	<10	20	<10
	I	> 2560	640	640	320
	II	80	<10	20	<10
	III	40	<10	20	<10
	I	> 2560	> 2560	640	320
	II	160	40	20	<10
	III	80	<10	<10	<10

^{1/} Reciprocal of 50% plaque reduction titer vs. indicated serotype.

Table 23. (Continued)

Case No.	Fraction No.	Antibody Titer _{1/}			
		D-1	D-2	D-3	D-4
789	I	1280	640	40	2560
	II	40	<10	<10	40
	III	20	<10	<10	<10
794	I	<10	640	> 2560	640
	II	<10	<10	20	<10
	III	<10	20	20	<10
795	I	320	160	<10	640
	II	<10	<10	<10	<10
	III	<10	<10	<10	<10

_{1/} Reciprocal of 50% plaque reduction titer vs. indicated serotype.

Beta_{1C/a} Globulin Levels in the Dengue Shock Syndrome

The immune response in cases of dengue shock syndrome results in the presence of anti-dengue Ig-G in the serum during the course of the viremia. Since Ig-G has complement-fixing properties, it seems probable that an antigen-antibody reaction, which interacts with serum complement, takes place in vivo during the course of the illness. In a preliminary effort to obtain further information on complement components during the course of dengue shock syndrome serum Beta_{1C/a} globulin levels were serially measured on 23 patients.

Quantitative measurements of serum Beta_{1C/a} were done by the single radial diffusion method using "Immunoplates" (obtained from Hyland Laboratories) containing anti-Beta_{1C/a} antibody. Reference standards for Beta_{1C/a} were also obtained from Hyland Laboratories.

To determine normal levels of Beta_{1C/a} globulin in the pediatric population in Bangkok, serum from 45 normal children ages 1 through 7 were tested. The mean Beta_{1C/a} level for this group was 185 mg% with a range from 125-265 mg%. This mean is slightly higher than the mean of 145 mg% obtained for American adults.

Cases of dengue shock syndrome hospitalized in Children's Hospital, Bangkok were studied. Criteria for inclusion in the study included serologic evidence of dengue virus infection, fever, hypotension (systolic pressure less than 90), elevated hematocrit and, in most cases a petechial or hemorrhagic rash. Sera were separated within 3 hours of collection and stored at -70°C prior to testing.

The results obtained are summarized in table 24. In every case the serum Beta_{1C/a} level was depressed during the acute phase of the illness. In only one case (#770) did the level remain within the limits of the normal range and in that case a marked reduction on days 5 and 6 was noted. In seven cases, levels of less than 50 mg% were noted. In 16 cases in which a late convalescent serum was obtained 25 or more days after the onset of illness the Beta_{1C/a} level had returned to normal levels. In these cases the mean convalescent Beta_{1C/a} level was 185 mg%, a figure similar to the mean of the normal control children. Early convalescent serum i.e. from 16-25 days after onset were usually within the normal range but in some instances moderately depressed.

The results clearly indicated a lowering of serum Beta_{1C/a} levels during the acute phase of the dengue shock syndrome. Since this globulin is related or identical to the third component of complement it appears that marked changes involving the complement system occur during the course of dengue shock syndrome. The mechanism which causes the reduction in the serum Beta_{1C/a} levels is not yet known. Possibilities include depression of synthesis of the globulin, removal from the serum by combining with antigen-antibody complexes, or loss from the serum as the result of increased vascular permeability. In the light of present knowledge concerning the immunologic phenomenon occurring during the course of this illness it seems likely that the observed results are caused by the combining of Beta_{1C/a} globulins with antigen-antibody complexes in vivo.

able Serum $\beta_{1c/a}$ globulin levels and antibody titers patients with dengue shock syndrome

Case No.	Day After Onset	Dengue Antibody _{1/f}	Beta _{1c/a2/f}
	(s) ₃	10240	
		20480	140
			140
		320	
	19	1280	
	33	1280	180
		20480	105
		20480	165
		160	
		20480	105
		20480	
		20480	80
		20480	100
		2560	
		5120	
		5120	165
		1280	
		2560	125
		2560	

24. (Continued)

<u>Case No.</u>	<u>Day after Onset</u>	<u>Dengue HI antibody₁/</u>	<u>Beta_{1c}/a₂/</u>
	3 (s)	320	58
	4 (s)		33
	6		47
	16	10240	155
	31	5120	194
	3 (s)	640	95
	4 (s)	1280	65
	5	2560	80
	6		80
	13		250
	29		240
	10 (s)	640	140
	11 (s)	2560	62
	12 (s)	2560	65
	13		72
	16		194
	19		215
770	4 (s)	2560	165
	5 (s)		125
	6		125
	12	20480	180
	18	10240	215
	5 (s)	2560	95
	6	5120	80
	7	5120	112
	8		155
	17		165
	31		174
	5 (s)	80	36
	6 (s)	320	25
	7	640	34
	8		47
	9		52
	11		112
	4 (s)	20	165
	5 (s)	160	125
	6 (s)	640	80
	7		95
	8		105
	18		180
827	6 (s) ₂ /	320	47
	7		125
	8		105
	9		125
	21	1280	165
	30	640	180

Table 24. (Continued)

<u>Case No.</u>	Day after onset	Dengue HI antibody ^{1/}	Beta _{1c/a} ^{2/}
	4 (s)	160	80
	5 (s)		62
	6		80
	7		140
	17	1280	180
	28	5120	240
	4 (s)	80	80
	5		47
	6		37
	7		74
	19	1280	240
	33	2560	180
	6 (s)	1280	42
	7		72
	8		105
	9	2560	140
	5 (s)	160	95
	6		68
	7		80
	8		105
	21	10240	180
	37	5120	180
837	5 (s)	320	95
	6		62
	7		72
	8		105
	17	1280	180
	31	1280	180
	5 (s)	320	62
	6		65
	7		72
	8		80
	14	5120	140
	28	5120	155

^{1/} Reciprocal of titer vs. 4 units of dengue-1 antigen.

^{2/} Mg./100 ml.

^{3/} (s) = days on which shock was observed.

A Plaque Assay Method for Tembusu Virus

The presence of Tembusu virus in Thailand has been demonstrated by recovery of four Tembusu virus strains from Culex gelidus and Culex tritaeniorhynchus mosquitoes captured at Bang Phra, Chonburi. It became necessary to develop accurate and efficient laboratory methods for infectivity assays and neutralizing antibody measurements.

Tembusu virus strains were found to produce very hazy and usually uncountable plaques in LLC-MK₂ cell cultures when the routine method for producing dengue and JE plaques was used. Preliminary experiments indicated that reduction of the concentration of amino acids and vitamins in the overlay media improved the clarity of Tembusu virus plaques. The experiments reported herein were carried out in an attempt to determine the optimal conditions for plaque production by Tembusu in LLC-MK₂ cell cultures and to develop a plaque reduction neutralization test method. For these experiments the Malaysian prototype strain (MM 1775) of Tembusu virus was used at the 10th suckling mice passage level. The results of varying concentrations of glutamine, amino acids and vitamins and calf serum on plaque size and clarity are shown in Table 25. The medium was made in Hank's balanced salt solution with 1% Noble agar, 0.02% DEAE dextran, antibiotics, plus amino acids (Eagles), vitamins (Eagles) and inactivated (56°C 30 min) calf serum as noted. A second overlay containing neutral red was added after 5 days and the plaques read on the following day. Concentrations used in experiment number 1 are those routinely used for plaque assay of dengue, JE, chikungunya and sindbis viruses. This combination of ingredients in the overlay media resulted in the production of small plaques with very poor definition. A 50% reduction in amino acids and vitamins resulted in marked improvement in both plaque size and clarity when 5% calf serum was used. Further reduction in amino acids and vitamins decreased plaque size. The optimal results obtained in this series of experiments were seen in experiment number 2 and plaque size and clarity was judged adequate for accurate plaque assays and plaque reduction neutralization tests. The clarity of plaques produced by JE and dengue viruses was also improved by reducing the amino acid and vitamin concentration in this manner.

Plaque reduction neutralization tests were carried out in one-ounce bottles out as previously described for the dengue viruses using the above described modification of the overlay media. Tembusu virus was compared with other group B arboviruses known to be present in Thailand. Results, shown in table 26 indicate clear antigenic difference and offer convincing evidence of the usefulness of the plaque reduction neutralization tests for identification of agents in this group. In spite of the fact that hyperimmune antiserum were used in several instances differences, in heterologous and homologous titers were greater than eight-fold for all agents tested.

Trial of Heparin-Manganous Chloride Treatment of Sera for Hemagglutination-Inhibition Tests

Heparin-manganous chloride treatment of sera has been shown by other investigators to remove Beta lipoproteins from sera and has been used for removal of non-specific inhibitors of Reovirus hemagglutinin. The heparin-manganous chloride method was tested in comparison with the acetone extraction method to determine the suitability for use in arbovirus diagnostic tests.

For heparin-manganous chloride treatment, 0.05 ml of 1.0 M manganous chloride and 0.04 ml of heparin solution (5000 units/ml) was added to inactivated serum diluted 1:10 in borate-saline. The mixture was allowed to stand for 20 minutes at 4°C and then centrifuged at 2000 rpm for 10 minutes to remove the precipitate. Table 27 shows the results of comparative HI tests of untreated, acetone treated, and heparin-manganous chloride treated serum from patients. In several instances, heparin-manganous chloride treatment failed to remove non-specific inhibitors which were removed by acetone treatment. In addition, retesting of heparin-manganous chloride treated sera after storage for six day at 4° showed marked increase in titers in several instances possibly due to reactivation of non-specific inhibitors.

The immunoglobulin content of six sera were tested prior to treatment and following acetone or heparin-manganous chloride treatment. Results, summarized in table 28, indicate that the two methods are comparable and in most instances immunoglobulin levels following treatment are only slightly reduced from pre-treatment levels.

Table 25. Effect of varying concentrations of amino acids, vitamins and calf serum in overlay medium^{1/} on plaque formation of Tembusu virus in LLC—MK₂ cell culture.

Expt No.	Concentration in Percent				Infectivity Titer pfu/0.3 ml	Plaque Size (mm)	Plaque Definition
	Glutamine	Amino Acid Soln. (Eagles)	Vitamin Soln. (Eagles)	Calf Serum			
1.	0.3	1.0	1.0	10.0	5 x 10 ⁷	1.0	poor
	0.3	1.0	1.0	5.0	5 x 10 ⁷	1.0	poor
2.	0.15	0.5	0.5	5.0	2 x 10 ⁷	2.0	good
	0.15	0.5	0.5	2.0	3 x 10 ⁷	2.0	fair
3.	0.075	0.25	0.25	5.0	1.8 x 10 ⁷	1.0	good
	0.075	0.25	0.25	2.0	1.5 x 10 ⁷	1.0	good

^{1/} Hank's BSS with 1% Noble Agar, .02% DEAE dextran, pH 8.3 and other ingredients as indicated.

Table 26. Comparison of Tembusu virus with the group B agents of Thailand by neutralization tests.

Tembusu Virus vs.		Tembusu Antiserum vs.	
Antisera	Titer Ht /Ho ^{1/}	Virus	Titer Ht /Ho ^{1/}
dengue-1	20/320	dengue-1	240/18000
dengue-2	<10/640	dengue-2	40/18000
dengue-3	<10/350	dengue-3	20/18000
dengue-4	20/190	dengue-4	140/18000
JE	400/3500	JE	<10/18000
Wesselsbron	<10/1600	Wesselsbron	20/18000
Tembusu	18000		

^{1/} Reciprocal of 50% plaque reduction titers, heterologous/homologous

Dengue Cross Protection Studies in Gibbons

(Hylobates lar)

A study has been initiated to determine the degree and duration of viremia following subcutaneous inoculation of dengue viruses in gibbons and to determine the extent of heterotypic immunity to heterologous dengue virus challenge. An attempt will be made to correlate humoral antibody with immunity.

Eight gibbons were inoculated subcutaneously with 0.5 ml. of dengue-2 virus suspension (BKM-1749, LLC-MK₂ passage-1) containing 1.6×10^3 pfu. Blood samples were collected daily for 14 days for viremia assay and at 1 and 3 months for antibody determinations.

All eight gibbons developed viremia with the primary dengue-2 infection. Virus was first detectable on the day following infection and persisted for an average of eight days. One gibbon was viremic for 12 days. Precise quantification of viremia has proved difficult due to the presence of non-specific virus inhibitory activity of the sera. In many instances, virus was undetectable by direct plaque assay but could be detected using the delayed plaque method. In some instances no infectious virus was detectable at low dilutions of serum but could be found at higher dilutions up to $10^{-2.5}$. Reporting of viremia levels in these animals will await completion of additional tests.

Four of the gibbons were challenged with a heterologous dengue virus three months following the dengue-2 infection. Two gibbons, S8 and S27, were inoculated subcutaneously with 2.2×10^4 pfu of dengue 1 virus (BKM-725, LLC-MK₂ passage 3). Two gibbons, S9 and S70, were inoculated with 1.6×10^3 pfu of dengue-4 virus (29676, LLC-MK₂ passage 2). Results of viremia tests and HI antibody tests following the heterologous challenge are shown in table 29. Gibbon S8 had no HI antibody to dengue-1 antigen when inoculated and viremia was detected on days 2, 4, 5 and 7 post infection. Gibbon S27 had dengue-1 HI antibody at the time of inoculation and in this animal no viremia was detected. In gibbon S8, in which a viremia was noted HI antibody titers increased over 8-fold to all dengue antigens, however, only a 2-fold increase by day 10 was noted in the gibbon, S27, in which viremia did not occur.

The two gibbons inoculated with dengue-4 developed viremia and a rise in HI antibody was seen in both by day 10. In gibbon S70 a significant drop in HI antibody titers occurred during the period of viremia followed by a rise to above pre-infection levels.

It is apparent that, in this experimental situation, gibbons previously infected with dengue-2 virus may be susceptible to dengue-1 and dengue-4 infection as early as 3 months following the primary infection. Additional experiments are in progress to determine susceptibility to a third dengue serotype and to determine the duration of heterologous protection. In the gibbons in which viremia developed the decrease in HI antibody noted may be due to absorption of antibody by circulating virus.

Summary

Results of identification of dengue viruses from Thailand, Vietnam, Malaysia and Tahiti were reported. Preliminary identification and subsequent comparative studies were done by plaque reduction neutralization tests. Evidence for antigenic variation within dengue serotypes was noted.

Table 27. Comparison of HI titers of untreated, acetone extracted and heparin-MnCl₂ treated human convalescent sera and normal human sera.

Sera	Dengue-1			Jap. Enceph.			Chikungunya		
	Untreated	Acetone	MnCl ₂	Untreated	Acetone	MnCl ₂	Untreated	Acetone	MnCl ₂
D-1	> 1280 ^{1/}	640	> 1280	> 1280	> 1280	640	80	0	20
D-1	320	20	80	320	0	0	80	0	20
JE	> 1280	> 1280	> 1280	> 1280	320	160	40	0	20
JE	160	40	80	80	40	40	320	80	80
Chik	80	0	0	80	0	0	160	40	80
Neg	160	0	40	160	0	0	40	0	20
Neg	80	0	0	80	0	0	40	0	20

^{1/} Reciprocal of titer vs. 4-8 units HA antigen.

39

Table 28. Immunoglobulin levels^{1/} of 1: 10 dilutions of human sera after acetone extraction or heparin-MnCl₂ treatment.

Human Sera	Ig-G			Ig-A			Ig-M		
	Untreated	Acetone	MnCl ₂	Untreated	Acetone	MnCl ₂	Untreated	Acetone	MnCl ₂
1	140 ^{2/}	120	140	29	20	29	16	18	16
2	140	85	120	32	20	29	10	13	11
3	45	25	40	31	26	26	13	14	13
4	95	75	95	40	34	36	40	25	40
5	140	150	140	42	38	41	16	20	20
6	160	140	140	31	28	29	14	13	14

^{1/} Determined by single radial diffusion assay.

^{2/} mg. %.

Table 29. Viremia and HI antibody response in gibbons challenged with a heterologous dengue virus three months after a dengue-2 infection.

Gibbon No.	Challenge Virus	Day After Inoculation	Viremia	HI Titer vs.			
				D-1			D-4
S8	dengue-1 2.2 x 10 ⁴ pfu	0	nt _{1/}	<20 _{2/}	160	40	80
		1	nt	20	40	20	80
		2	10 ^{-2.5}	<20	160	20	40
		3	nt				
		4	10 ^{-2.5}	20	160	20	40
		5	10 ^{-1.0}				
		6	neg				
		7	10 ^{-1.0}				
		8	neg				
		9	neg				
S27	dengue-1 2.2 x 10 ⁴ pfu	10	neg	320	1280	640	640
		0	nt	40	320	80	160
		1	nt	20	320	80	80
		2	neg	20	160	40	160
		3	neg	20	320	80	160
		4	neg	20	320	80	80
		5	neg				
		6	neg				
		8	neg				
		9	neg				
S9	dengue-4 1.6 x 10 ³ pfu	10	neg	80	640	160	160
		0	nt _{1/}	<20 _{2/}	80	20	20
		1	nt	<20	80	<20	<20
		2	10 ^{-2.5}	<20	40	<20	<20
		3	10 ^{-2.5}	<20	40	<20	<20
		4	10 ^{-2.5}	<20	80	<20	<20
		5	10 ^{-2.5}				
		6	neg				
		7	neg				
		8	neg				
S70	dengue-4 1.6 x 10 ³ pfu	9	neg				
		10	neg	80	320	320	320
		0	nt	20	160	80	160
		1	nt	20	80	40	80
		2	neg	20	80	40	80
		3	10 ^{-1.0}	<20	80	20	80
		4	10 ^{-2.5}	<20	40	40	80
		5	neg				
		6	neg				
		7	neg				
8	neg						
9	neg						
10	neg	40	160	160	320		

1/ Highest dilution in which virus detected, nt = not tested.

2/ Reciprocal of titer vs. 8 units of indicated antigen.

Plaque size variation has been observed in strains of dengue-2 viruses. Pure clones of large and small plaque variants were obtained from the BKM-551 strain of dengue-2. Both variants were neutralized by dengue-2 antisera.

A micro method for performing plaque reduction neutralization tests for dengue and JE viruses was described.

Immunochemical studies of the antibody response in dengue shock syndrome indicate that the predominant antibody formed in shock syndrome patients was Ig-G which had virus neutralizing, hemagglutination-inhibiting, and complement-fixing activity against dengue antigens. This antibody was present in high titer during the acute phase of the illness. Serial studies of Beta_{1c/a} globulin levels were carried out in patients with dengue shock syndrome and results indicated that a significant decrease in Beta_{1c/a} globulin occurred concomitantly with the shock phase of the illness followed by a return to normal levels during convalescence.

Methods for plaque assay of Tembusu virus and assay of neutralizing antibody by plaque reduction neutralization test were described using LLC-MK₂ cell culture. The metabolic content of the overlay media was a critical factor for plaque formation.

Preliminary studies of cross protection between dengue viruses in gibbons were carried out. Viremia occurred in primary dengue-2 infections in antibody free gibbons. Subsequent infection 3 months later with dengue-1 or dengue-4 viruses also resulted in viremia.