

## 2. Title: Laboratory Studies on Arboviruses

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### OBJECTIVE

1. To develop a laboratory animal model for sequential dengue infections, in order that immunologic and clinical responses can be observed and related to those occurring during infection in humans.
2. To determine the relative potential of A. aegypti, A. albopictus, and A. scutellaris to become infected with dengue viruses under experimental conditions, and to develop simple, efficient and reproducible techniques for experimental dengue infection of Aedes mosquitoes.
3. To investigate biologic properties of plaque variants of dengue viruses and to relate plaque morphology to growth of these viruses in tissue culture and in experimental animals.
4. To determine the characteristics of the growth curve of Wesselsbron virus in LLC—MK2 cells, as a preliminary step toward further laboratory definition of this virus.

### DESCRIPTION

1. Dengue cross—challenge experiments have been conducted in gibbons and the extent of cross immunity has been measured at 6 and 12 weeks after infection. Methods and preliminary experiments were described in last years report.

2. Mosquito infection studies have included efforts to infect mosquitoes with dengue viruses using the membrane feeding technique, and comparing variables such as source of virus, species of mosquito, and ingredients of feeding solutions.
3. Large and small plaque variants of dengue-2 virus, derived from a pool of infected A. aegypti mosquitoes, were studied for evidence of distinguishing features; the methods used for purification and passage of these strains were described previously. Experiments concerned with antigenicity and growth characteristics in tissue culture and experimental animals are in progress.
4. Work with Wesselsbron virus is preliminary, and is concerned with growth in LLC-MK2 cells.

## PROGRESS

### Sequential Dengue Infections in Gibbons

Gibbons (Hylobates lar) have been infected sequentially with several serotypes of dengue viruses, and the course of each infection followed with clinical observations, determination of viremias, and antibody responses. Gibbons were either splenectomized young adults, or intact juvenile animals. Dengue viruses were low tissue culture strains derived from mosquitoes (types 1,2 and 4) or from human serum (type 3). Gibbons received approximately 1000 PFU of virus subcutaneously, following which blood samples were obtained daily for 10 days for viremia studies and at 2,4, and 10 weeks for antibody studies. Virus isolation techniques and HI and neutralization tests were as described in previous reports. Rectal temps, pulse, and blood pressures were recorded daily for 10 days in juvenile animals, and white blood cell counts and hematocrits were measured. Gibbons were challenged after an interval of either 10 or 12 weeks after primary infection, and again after 12 weeks after secondary infection.

#### Infection in splenectomized gibbons

Dengue 1 virus. (Table 1). Eight gibbons were infected and all developed a viremia after primary infection. After 12 weeks all the gibbons were challenged but none developed a second viremia. However all six gibbons challenged with a heterologous virus showed a marked rise in antibody titer, whereas no antibody response was noted in the two gibbons challenged with homologous virus. When challenged for a second time after a further 12 weeks one gibbon (no. 93) developed a viremia.

Dengue 2 virus (Table 2) Seven gibbons were infected with dengue-2 virus and all developed a viremia. After 12 weeks the gibbons were challenged and the gibbons challenged with either dengue-1 virus or dengue-4 virus developed a second viremia. Two gibbons were also challenged with dengue-3 virus and two with homologous virus but none of these developed a second viremia, although both gibbons challenged with dengue-3 virus showed a marked rise in HI-antibody titer. After a further interval of 12 weeks the five gibbons previously challenged with a heterologous virus were challenged for a second time and one gibbon (no. 9) developed a third viremia.

Because both of the gibbons challenged with dengue-4 virus had been readily reinfected, a second group of two gibbons (No. 13 & 19) were infected with dengue-2 virus and challenged with dengue-4 virus after an interval of six weeks. Both of these animals developed a second viremia but in this case virus could only be isolated from serum by using the delayed plaque technique. Both of these gibbons were challenged for a second time with dengue-1 virus after an interval of 12 weeks, and both developed a third viremia.

One of these gibbons (no. 19) died on the ninth day after infection and virus was isolated from liver, lung and mesenteric lymph node as well as from serum. This gibbon had become febrile to 106°F (normal 101°-104°F) on the fourth day after infection and became progressively weaker until it died on the ninth day. On autopsy, hemorrhagic lesions were found in the heart muscle, lungs and stomach lining. Pathologic studies, however, revealed changes thought to be more consistent with an acute lymphomatous leukemia and this was considered to be the most likely cause of death. The other two gibbons that received this inoculation sequence remained well and no suggestive lesions were found in gibbon No. 13 when it was sacrificed 13 days after infection, and examined microscopically.

TABLE 1

Gibbons infected initially with dengue-1 virus.

Gibbon No. & sex	Original infection			First challenge			Second challenge			
	Inoculum dose (pfu)	Occurrence of viremia <sup>1</sup>	Interval after 1st infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>2</sup>	Interval after 2nd infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>3</sup>
23 ♂	D1(6×10 <sup>2</sup> )	5-7	12 week	D1(5×10 <sup>2</sup> )	0	0	12 weeks	NT		
25 ♂	"	5-7	"	"	0	0	"	NT		
80 ♀	D1(35)	7	"	D2(10 <sup>3</sup> )	0	>32	"	D3(30)	0	8
82 ♀	"	5-9	"	"	0	128	"	D4(3×10 <sup>2</sup> )	0	4
83 ♂	"	5-9	"	D3(8×10 <sup>2</sup> )	0	8	"	D2(10)	0	16
84 ♀	"	5-7	"	"	0	4	"	D4(3×10 <sup>2</sup> )	0	16
93 ♀	"	5-8	"	D4(5×10 <sup>3</sup> )	0	64	"	D3(30)	6-7	16

1. Days on which viremia was detected.

2. Multiplicity of increase in titer between pre-infection titer and titer to challenge virus 2 to 4 weeks after infection.

3. Greatest multiplicity of increase in titer between pre-infection titer &amp; titer to the dengue serotype showing highest antibody rise 2 to 4 weeks after infection.

TABLE 2

Gibbons infected initially with dengue-2 virus.

Gibbon No. & sex	Initial infection		First challenge			Second challenge				
	Inoculum dose (pfu)	Occurrence of viremia <sup>1</sup>	Interval after 1st infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>2</sup>	Interval after 2nd infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>3</sup>
8 ♀	D2(1.6×10 <sup>3</sup> )	1-6 day	12 weeks	D1(2.2×10 <sup>4</sup> )	2-5	16 fold	12 week	D2(50)	0	0
9 ♀		1-7	"	D4(1.6×10 <sup>3</sup> )	2-5	16	"	D1(1.6×10 <sup>3</sup> )	3	2
13 ♂		2-6	6 weeks	D4(1.6×10 <sup>4</sup> )	4-5	32	"	D1(7×10 <sup>2</sup> )	7-8	8
19 ♀		2-8	"	D4(1.6×10 <sup>4</sup> )	5-7	16	"	"	7-9 <sup>3</sup>	NT
22 ♀		3-8	12 weeks	D3(5×10 <sup>2</sup> )	0	32	"	D1(3.3×10 <sup>2</sup> )	0	0
36 ♂		2-7	"	D3(5×10 <sup>2</sup> )	0	4	"	D4(7.5×10 <sup>2</sup> )	0	4
51 ♂		2-7	"	D2(6.6×10 <sup>2</sup> )	0	0	"	NT		
57 ♂		3	"	"	0	0	"	NT		
70 ♀		2-6	"	D4(1.6×10 <sup>3</sup> )	3-4	4	"	D3(7×10 <sup>2</sup> )	0	0

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1. Days on which viremia was detected.
2. Multiplicity of increase in titer between pre-infection titer and titer to challenge virus 2 to 4 weeks after infection.
3. Gibbon died on day 9.
4. Multiplicity of increase in titer between pre-infection titer and titer to the dengue serotype showing highest antibody rise 2 to 4 weeks after infection.

Dengue 3 virus (Table 3) Eight gibbons were infected and all developed a viremia after primary infection. After twelve weeks all the gibbons were challenged and one of the two gibbons challenged with dengue-1 virus and both of the gibbons challenged with dengue-2 or dengue-4 virus developed a second viremia. Neither of the two gibbons challenged with homologous virus developed a second viremia nor showed an antibody response to the infection, whereas all of the gibbons challenged with heterologous virus showed a marked HI antibody titer rise.

When challenged for a second time after a further 12 weeks one gibbon infected with a dengue 3-1-4 sequence developed a third viremia.

Dengue 4 virus (Table 4). All eight gibbons developed a viremia following primary infection. When infected after an interval of 12 weeks only one of the two gibbons in each of the heterologous challenge groups developed a second viremia but all the gibbons showed a rise in HI antibody titer. Neither of the gibbons challenged with homologous virus showed any evidence of infection.

#### Infection in intact juvenile gibbons

Dengue 2 virus (Table 5) All of the gibbons used in the above experiments had previously been splenectomized and used for malaria studies. Six juvenile intact animals were therefore infected with dengue-2 virus and all developed a viremia. After six weeks each was challenged with dengue-4 virus and three of the six developed a second viremia. Virus was isolated from gibbons No. 165 & 166 only by the use of the delayed plaque method. Five of the six gibbons showed a marked rise in HI antibody titer.

One day prior to the third infection two of the gibbons (Nos. 165 & 175) were injected with 2.0 ml of Bordetella pertussis vaccine in order to induce a leucocytosis. This injection was repeated on the second post-infection day. Although no significant leucocytosis developed in either animal, both of these gibbons developed a third viremia and the duration of viremia in these two animals was markedly greater than in the other four animals.

#### Pre infection serology

Six (9%) of the gibbons tested before inoculation were found to have pre-existing (HI) antibodies to at least one of the four dengue viruses. Of these gibbons 4 (7%) of 54 also had N antibodies to at least one of the dengue viruses indicating infection of these animals in nature. Thirty nine of the antibody-free animals were subsequently used in viremia studies.

#### Antibody response

A series of representative HI antibody responses to primary and secondary infection is shown in table 6 & 7. Serum was broadly crossreactive even after primary infection and HI antibody to all four types was present at the time of challenge. There was no apparent correlation between pre-infection HI antibody titer and the appearance of a second viremia. All the gibbons had circulating N antibody to both the initial and challenge viruses at the time of challenge, although the titer to the challenge virus was low in most cases (Table 8).

#### Mosquito Infectivity Studies

Mosquitoes were infected by allowing them to feed on dengue virus diluted in one of three feeding solutions (a) 5 ml of fetal bovine serum (inactivated 56° for 30 min) + 0.005 M ATP (b) 2.0 ml of packed gibbon R.B.C.'s + 3 ml of FBS & ATP. (c) 5.0 ml of citrated gibbon blood.

The feeding solutions were placed in a water-heated feeding bell with a Baudruche membrane attached. Mosquitoes were allowed to feed through the membrane for 1 hour after which the engorged mosquitoes were separated and held for the duration of the experiment. Any mosquitoes not obviously engorged were discarded. The feeding solutions were titrated in LLC MK<sub>2</sub> cells at the end of the feeding period.

Mosquitoes were killed using CO<sub>2</sub> or by freezing and held at -70°C until processed. Mosquitoes were triturated either singly or in pools of two using rubber vaccine bottle stoppers and plungers from 1ml tuberculin glass syringes and diluted in 2.0 ml of virus diluent. The suspension was spun at 10,000 rpm for 30 minutes and the supernatant fluid assayed for virus presence using both the direct and delayed plaque techniques in LLC-MK<sub>2</sub> cells.

TABLE 3

Gibbons infected initially with dengue-3 virus.

Gibbon No. & sex	Initial infection		First challenge			Second challenge				
	Inoculum dose (pfu)	Occurrence of viremia <sup>1</sup>	Interval after 1st infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>2</sup>	Interval after 2nd infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>3</sup>
53 ♂	D3 (5 x 10 <sup>2</sup> )	2	12 weeks	D1 (3.3 x 10 <sup>2</sup> )	0	8 fold	12 weeks	D2 (<10)	0	0
60 ♀	"	4-5	"	"	4-6	8	"	D4 (3 x 10 <sup>2</sup> )	1-4	4
77 ♀	D3 (6.6 x 10 <sup>2</sup> )	3-7	"	D3 (8 x 10 <sup>2</sup> )	0	0	"	NT		
78 ♀	"	2-5	"	"	0	0	"	NT		
81 ♀	D3 (5 x 10 <sup>2</sup> )	4-5	"	D2 (6 x 10 <sup>2</sup> )	5-6	16	"	D4 (3 x 10 <sup>2</sup> )	0	NT
90 ♀	"	2-7	"	"	3-4	16	"	D1 (<10)	0	0
92 ♀	"	2-6	"	D4 (7.5 x 10 <sup>2</sup> )	3-5	8	"	D1 (<10)	0	4
94 ♀	"	2-6	"	"	2-5	8	"	D2 (<10)	0	0

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1. Days on which viremia was detected.
2. Multiplicity of increase in titer between pre-infection titer and titer to challenge virus 2 to 4 weeks after infection.
3. Multiplicity of increase in titer between pre-infection titer and titer to the dengue serotype showing highest antibody rise 2 to 4 weeks after infection.

TABLE 4

Gibbons Infected initially with dengue-4 virus.

Gibbon No. & sex	Initial infection		First challenge			Second challenge				
	Inoculum dose (pfu)	Occurrence of viremia <sup>1</sup>	Interval after 1st infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>2</sup>	Interval after 2nd infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>3</sup>
61 ♀	D4(3.3x10 <sup>3</sup> )	2-5	12 weeks	D1(8.3x10 <sup>2</sup> )	0	8 fold	12 weeks	D2(10 <sup>3</sup> )	0	0
63 ♀	"	2-6	"	"	5-6	2	"	NT		NT
71 ♂	"	4-5	"	D2(1.6x10 <sup>3</sup> )	0	16	"	D3(10 <sup>3</sup> )	0	0
74 ♂	"	2-5	"	"	6	8	"	D1(3x10 <sup>2</sup> )	0	4
76 ♂	"	2-5	"	D3(1.6x10 <sup>3</sup> )	0	16	"	"	0	4
86 ♀	(5x10 <sup>3</sup> )	1-5	"	D2(20)	3-7	8	"	NT		
P2 ♀	(6.6x10 <sup>2</sup> )	2-6	"	D3(1.6x10 <sup>3</sup> )	0	32	"	D2(10 <sup>3</sup> )	0	4
P7 ♀	"	3-5	"	D4(3.3x10 <sup>3</sup> )	0	0	"	NT		
P10 ♂	"	2-6	"	"	0	0	"	NT		

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1. Days on which viremia was detected.

2. Multiplicity of increase in titer between pre-infection titer and titer to challenge virus 2 to 4 weeks after infection.

3. Multiplicity of increase in titer between pre-infection titer and titer to the dengue serotype showing highest antibody rise 2 to 4 weeks after infection.

TABLE 5

Juvenile gibbons infected initially with dengue-2 virus.

Gibbon No. & sex	Initial infection			First challenge			Second challenge			
	Inoculum dose (pfu)	Occurrence of viremia <sup>1</sup>	Interval after 1st infection	Inoculum dose (pfu)	Occurrence of viremia of infection	HI antibody response <sup>2</sup>	Interval after 2nd infection	Inoculum dose (pfu)	Occurrence of viremia	HI antibody response <sup>4</sup>
165 0	D2(4x10 <sup>2</sup> )	2-6	6 weeks	D4(?)	4,7	8 fold	12 weeks	D1(100)	5-8 <sup>3</sup>	16
166 0		1-7	"		8	18	"		0	16
167 0		1-7	"		0	4	"		0	32
169 0		2-6	"		0	16	"		0	16
173 0		1-7	"		0	0	"		5	16
175 0		1-6	"		1-4	4	"		2-6 <sup>3</sup>	32

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1. Days on which viremia was detected.
2. Multiplicity of increase in titer between pre-infection titer and titer to challenge virus four weeks after infection.
3. Received B. pertussis vaccine.
4. Multiplicity of increase in titer between pre-infection titer and titer to the dengue serotype showing highest antibody rise 2 to 4 weeks after infection.

TABLE 6

HI antibody response following primary infection

Gibbon	Infecting virus	Time <sup>1</sup>	HI antibody				Gibbon	Infecting virus	Time	HI antibody			
			D1	D2	D3	D4				D1	D2	D3	D4
80	D1	0	0 <sup>2</sup>	0	0	0	90	D3	0	0	0	0	
		4	40 <sup>3</sup>	0	40	0			20	20	20	80	80
		12	40	0	0	20			40	40	80	80	80
83		0	0	0	0		94	"	0	0	0	0	
		4	160	20	40	80			20	40	40	20	40
		12	160	40	40	80			0	0	20	20	20
8	D2	0	0	0	0		61	D4	12	0	0	40	
		2	0	40	0	0			0	0	0	0	
		4	80	320	80	640			0	0	0	0	0
		12	0	160	40	80			2	0	20	0	40
70	"	0	0	0	0		76		12	80	80	160	
		2	0	40	0	80			0	0	0	0	0
		4	40	320	80	1280			20	20	40	20	40
		12	20	160	80	160			12	20	40	40	40
165	"	0	0	0	0		P2	"	0	0	0	0	
		2	0	20	20	20			2	20	20	20	20
		4	20	160	40	40			12	40	40	40	160
		6	40	80	40	80							

1. Weeks after infection.

2. Less than 1/20.

3. Reciprocal of HI antibody titer.

TABLE 7

HI antibody response following secondary infection.

Gibbon of infection	Sequence of infection	Viremia	Time <sup>1</sup>				Gibbon of infection	Sequence	Viremia	Time					
			D1	D2	D3	D4				D1	D2	D3	D4		
80	D1-D2	-	0	40 <sup>2</sup>	0 <sup>3</sup>	0	90	D3-D2	+	0	20	20	80	40	
			2	40	20	20				2	80	80	160	80	
			12	160	640	80			80		8	320	320	320	320
83	D1-D3	-	0	160	40	40	94	D3-D4	+	0	0	0	40	40	
			2	320	160	80			160		2	160	320	640	80
			12	640	640	320			320		8	80	160	160	320
8	D2-D1	+	0	0	160	40	61	D4-D1	-	0	80	160	160	160	
			2	320	1280	640			640		2	40	80	80	160
			12	160	320	160			320		4	640	640	640	1280
70	D2-D4	+	0	20	160	80	76	D4-D3	-	0	20	40	40	40	
			2	40	160	160			320		2	NT	NT	NT	NT
			12	160	160	320			640		4	640	640	640	1280
165	D2-D4	+	0	40	80	40	P2	D4-D3	+	0	40	40	40	160	
			2	160	320	320			320		2	640	640	320	640
			4	320	640	640			640		4	320	640	1280	1280
	12	40	80	80	40			12	160	80	160	160			

1. Weeks after infection.
2. Reciprocal of HI antibody titer.
3. Less than 1/20.

TABLE 8

Antibody level to challenge virus at time of challenge.

Gibbon	Initial	Challenge	Pre-infection titer		Viremia
			neut.	HI	
82	D1	D2	125 <sup>1</sup>	20 <sup>2</sup>	0
83	"	D3	125	40	0
84	"	"	125	40	0
93	"	D4	125	40	0
8	D2	D1	25	<20	2-5 <sup>3</sup>
9	"	D4	15	20	2-5
13	"	"	50	40	4-5
19	"	"	25	80	5-7
22	"	D3	25	80	0
36	"	"	25	80	0
70	"	D4	30	80	3-4
77	D3	D3	> 125	1280	0
78	"	"	> 125	160	0
81	"	D2	5	20	5-6
90	"	"	10	20	3-4
92	"	D4	25	20	3-5
94	"	"	25	40	2-5
71	D4	D2	50	40	0
74	"	"	125	80	6
76	"	D3	25	40	0
P2	"	"	5	40	0

1. Reciprocal of 50% plaque reduction titer.

2. Reciprocal of HI antibody titer.

3. Days on which viremia occurred.

Salivary glands were dissected, washed in Hanks solution containing penicillin and streptomycin and triturated in the same manner as the mosquitoes. Normally salivary glands from two mosquitoes were pooled.

Comparison of feeding solutions. It was found that fresh citrated gibbon blood gave the best results both for the percentage of mosquitoes feeding and the percentage becoming infected. (Table 9)

Infection attempts with dengue-2 virus. Infection of mosquitoes with dengue-2 virus seems to depend on the source of the virus used, e.g. (1) BKM1749-MK<sub>2</sub> passage 2 would not infect either A. aegypti or A. albopictus (2) D2 NG (C) & MB passage 27 infected A. aegypti, A. albopictus and A. scutellaris, however passage of D2 NG (c) through two MK<sub>2</sub> cell passages lowered the infectivity of the virus for A. aegypti from 100% to 16%.

Infection attempts with dengue-3 virus. A low tissue culture passage strain of dengue-3 virus was used (24969-MK<sub>2</sub> passage 3). Infectivity was found to depend on the feeding solution used. If heat-inactivated fetal bovine serum + 0.005 MATP was used A. albopictus was readily infected whereas it was difficult to infect A. aegypti. However if fresh citrated gibbon blood was used both species were readily infected.

Virus replication in mosquitoes. It was reported last year that following infection of A. aegypti and A. albopictus with dengue 2 virus (BKM1749 gibbon passage), the virus went into eclipse on the day following infection but reappeared on the second day after infection. The virus titer in the mosquitoes was found to increase until the fifth day when it became stationary. Further experiments using a different D2 virus strain (D2NG (C)) have shown that small amounts of virus can be detected even on the first day after infection in some mosquitoes and that all mosquitoes are infected by the fifth day after infection (Table 10).

Dengue transmission studies. At present the only way to demonstrate transmission of dengue virus by mosquitoes is to use human volunteers. Monkeys and gibbons are potential hosts for such experiments but are difficult to obtain in sufficient numbers. For these reasons a laboratory method for demonstrating transmission is desirable. It is known that arboviruses multiply in the salivary glands of mosquitoes and are excreted in the saliva at the time of feeding. Salivary glands have therefore been dissected daily from the 12th day after infection and assayed for virus presence. Although the presence of virus in the salivary glands does not prove ability to transmit, it can be assumed that absence of virus indicates a lack of ability to transmit. It has been found that virus is present in the salivary glands of most A. aegypti by the 13th day after infection whereas it is not present in an equivalent number of A. albopictus until the 16th day after infection. This points to a possible difference in the potential of the two species as vectors of dengue virus.

An attempt to demonstrate transmission of dengue viruses from infected suckling mice to A. aegypti mosquitoes. Suckling mice were inoculated either IC or IP with MK2 cell-derived or mouse brain-derived dengue virus (types 1-4) and A. aegypti were induced to feed on the mice 42, 48, 66, 72 and 84 hours post-inoculation. Engorged mosquitoes were then triturated and tested for dengue infection using the standard MK2 plaque system. The following observations were made: a. Low tissue culture passage viruses used in the experiments failed to produce illness or death in suckling mice after IC inoculation. b. No evidence was found for transmission of dengue virus from mice to mosquitoes, although mosquitoes were fed on mice during the course of a fatal infection in mice following IC or IP inoculation of mouse adapted virus. In one experiment, mosquitoes were fed on sick mice on the 4th day of infection after IC inoculation of mouse adapted virus. Five mosquitoes were triturated individually on days 0, 3, 7, 10, & 13 post feeding; no virus was detected in any mosquito.

Table 9. Membrane Feeding Studies in Aedes Mosquitoes

Virus	Feeding Soln	Virus ingested	Days Held	Results	
				A. aegypti	A. albopictus
D2 BKM	F.B.S.+0.005 M	93 pfu*	11	0/27	0/40
1749 P-2	ATP	9 pfu	11	0/48	0/57
		20	8	0/12	0/13
	Gibbon RBC+	200	8	0/10	0/22
	F.B.S. +ATP				
	Citrated gibbon blood	500	8	0/12	0/18
D2 NG (c)	FBS+ATP	25	9	2/5	n.t.
		3	11	0/6	1/6
SMB P-27		11	15	n.t.	2/5
	Gibbon RBC+	5	8	3/6	3/6
	Serum				
	+ATP	11	11	4/6	5/6
	Gibbon Blood	20	10	6/6	6/6
D2 NG (c)	Gibbon blood		10	3/19	n.t.
MK <sub>2</sub> P-2					
D3	F.B.S.+A.T.P.		10	0/24	3/17
24969-P3		1	11	1/50	19/48
	Gibbon RBC	0.1	10	0/1	1/3
	+F.B.S.+ATP				
	Gibbon blood	50	10	6/8	6/6

\*Amount ingested calculated from titration of feeding soln.

Table 10. Replication of D2(NGC) in Aedes Mosquitoes

Day	<u>A. aegypti</u>		<u>A. albopictus</u>	
	Body	Salivary gland	Body	Salivary gland
1	6/17	—	5/15	—
2	5/18	—	—	—
3	10/12	—	3/3	—
4			—	—
5	6/6	—	3/3	—
7				
8	3/6	—	3/6	—
10	3/4	0/4	12/12	0/6
12	3/3	0/3	—	—
13	4/4	8/10	—	—
14	6/6	12/12	—	1/4
15			—	1/6
16			—	4/6
17			—	5/6

Table 11. Virus isolation from mosquitoes collected at Bang Phra, Apr 68—Mar 69

Mosquito Species	Number of Pools tested	Number of mosquitoes tested	Positive Pools	Virus Identification
<i>Ae. catusticta</i>	10	23	0	0
<i>Ae. albopictus</i>	16	36	0	0
<i>Ae. lineatopennis</i>	38	211	0	0
<i>Ae. mediotineatus</i>	106	1800	0	0
<i>Ae. vexans</i>	224	4531	1	8
<i>Ae. vigilax</i>	64	617	0	0
<i>Arm. subalbatu</i>	73	380	0	0
<i>An. aconitus</i>	4	35	0	0
<i>An. campestris</i>	1	1	0	0
<i>An. philippinensis</i>	6	15	0	0
<i>An. subpictus</i>	38	205	0	0
<i>An. vagus</i>	19	85	0	0
<i>An. vamxayi</i>	3	3	0	0
<i>An. hycranus group</i>	4	6	0	0
<i>C. annulus</i>	125	1236	1	Tembusu
<i>C. bitaeniorhynchus</i>	35	97	0	0
<i>C. fuscocephalus</i>	45	425	0	0
<i>C. gelidus</i>	315	6539	0	0
<i>C. lutziafuscatus</i>	35	100	0	0
<i>C. nigropunctatus</i>	17	50	0	0
<i>C. pseudovishnui</i>	417	7158	1	Sindbis
<i>C. quinquefasciatus</i>	1509	35186	0	0
<i>C. sinensis</i>	11	30	0	0
<i>C. sitiens</i>	911	20113	1	Tembusu
<i>C. tritaeniorhynchus</i>	61	779	0	0
<i>C. whitmori</i>	14	30	0	0
<i>M. annulifera</i>	41	187	0	0
<i>M. crassipes</i>	336	4492	0	0
<i>M. indiana</i>	5	16	0	0
<i>M. uniformis</i>	174	2277	0	0
<i>M. mixed</i>	1	5	0	0
<i>C. mixed</i>	1	4	0	0
Total	4659	86672	4	71

Virus isolations from mosquitoes collected at Bang Phra: Apr 68—Mar 69

An extensive description and review of the Bang Phra study on the ecology of Japanese encephalitis virus, which began in 1966 and was terminated this year, is found in last year's annual report. A description of the final phases of the entomological studies is found in the Study on Mosquitoes section of this report. Table 11 depicts the number of mosquito pools tested for virus content in support of these field studies, and records the viruses recovered from infected pools. Each mosquito pool shown was tested by IC inoculation into two litters of suckling mice, followed by two passages of mouse brain into additional mice. Virus isolates were identified by means of plaque reduction test in MK2 cells.

Plaque Variants of Dengue Virus

During the course of virus isolation attempts from mosquitoes collected on Koh Samui during the past several years, it has been observed that there is considerable heterogeneity of plaque size of certain dengue strains. Large and small plaque variants of one such strain (BKM 551) have been selected and propagated according to techniques described in last year's report. These variants, designated LP-BKM 551 and SP-BKM 551, have been studied during the past year in an attempt to define some of their antigenic and biologic characteristics.

Serologic reactions of LP-BKM 551 and SP-BKM 551 were tested in the standard MK2 plaque reduction test with monkey antisera prepared against dengue 1-4 prototype strains. Results of a typical experiment are shown in Table 12. While LP-BKM 551 can clearly be identified as dengue-2 on the basis of this test, SP-BKM 551 is less well neutralized by dengue-2 antiserum. Neither virus is neutralized to any significant degree by antisera against the other serotypes. Likewise, these viruses do not react with antisera prepared against other known arboviruses of Thailand.

Table 12

Neutralization of small plaque (SP-BKM 551) and large plaque (LP-BKM 551) variants by dengue prototype antisera.

Virus	Antiserum			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4
SP-BKM 551	10/400*	70/640	20/300	20/300
LP-BKM 551	20/400	300/640	20/300	20/300

\* Numerator = titer of serum against indicated virus.  
Denominator = titer of serum against homologous virus.  
(reciprocal of 50% plaque reduction titer)

Table 13

Neutralization of LP-BKM 551, SP-BKM 551, BKM 551, and dengue-2 NG-C viruses

Virus	Antiserum			
	LP-BKM 551	SP-BKM 551	BKM 551	24742
LP-BKM 551	200 <sup>1</sup>	200	300	300
SP-BKM 551	40	70	400	70
BKM 551	NT	NT	160	100
NG-C	320	450	640	640

1. reciprocal of 50% plaque reduction titer
2. not tested

Table 14

Neutralization of dengue 1-4 prototype strains by SP-BKM 551 and LP-BKM 551 antisera

Virus	Antiserum	
	SP-BKM 551	LP-BKM 551
Dengue 1	<20*	20
Dengue 2	450	320
Dengue 3	<20	<10
Dengue 4	<20	20

\* reciprocal 50% plaque reduction titer

Table 15

Mortality in suckling mice after IC inoculation of SP-BKM 551 and LP-BKM 551 viruses

Virus	Mortality in mice		
	First passage	Second passage	Third passage
SP-BKM 551	0/16*	0/16	0/16
LP-BKM 551	16/16	16/16	16/16

\* number dead/number inoculated

Table 16

Resistance of suckling mice to dengue-2 challenge 21 days after IC inoculation of SP-BKM 551

Virus dilution	PFU/0.02 ml	Mortality after IC inoculation	Mortality after challenge	% resistant to challenge
-1	530	0/8*	0/8	100
-2	53	0/8	1/8	87.5
-3	5	0/8	1/8	87.5
-4	0	0/8	8/8	0
-5	0	0/8	8/8	0

\* number dead/number inoculated

In an attempt to explain the difference observed in the neutralization reaction of LP-BKM 551 and SP-BKM 551 viruses with dengue-2 antiserum, antisera to each were prepared in monkeys and these, as well as antisera against a strain of dengue-2 isolated from human serum (24742) and the parent BKM 551 strain, were tested in the plaque reduction test against LP-BKM 551, SP-BKM 551, BKM 551, and the New Guinea "C" prototype of dengue-2. Results are shown in Table 13. Antisera prepared against LP-BKM 551 and SP-BKM 551 are similar in that their reactivity is the same with NG-C strain and in SP and LP cross reactions. This result indicates that the poor neutralization of SP-BKM 551 virus with dengue-2 antiserum is probably not due to altered antigenicity. In fact, SP-BKM 551 is relatively poorly neutralized in this test by antiserum prepared against itself, which is more potent against LP and NG-C strains. In contrast, SP virus is well neutralized by antiserum prepared against the parent BKM 551 virus. Antisera prepared against both LP and SP-BKM 551 are type-specific, as illustrated in Table 14, which shows the results of testing these sera in the plaque reduction test against strains representative of each of the four dengue serotypes.

Additional studies are in progress to expand these preliminary observations on the serologic relationships of these two variants.

Studies are underway to determine the pathogenicity in suckling mice of SP-BKM 551 and LP-BKM 551. Low LLC-MK2 passage stocks of these viruses have been inoculated IC into 1-2 day old mice; in one experiment the estimated dose of SP-BKM 551 was 250 PFU, while other mice received approximately 40 PFU of LP-BKM 551. Results are shown in Table 15. None of 16 mice inoculated with SP-BKM 551 became sick during 10 days of observation and passage of a 10% suspension of brain taken on day 10 failed to induce illness on second or third passage. In contrast, all mice receiving LP-BKM 551 became sick, and those not sacrificed to prepare infected brain suspension, subsequently died. No loss of virulence was noted during three passages in mice.

Following the demonstration that SP-BKM 551 causes no illness in newborn mice, an experiment was performed to determine whether evidence could be detected for infection in these mice. A titration of SP-BKM 551 was performed in mice along with a simultaneous plaque titration in LLC-MK2 cells. No mice became ill during 21 days of observation. On day 21, mice were challenged with dengue-2 New Guinea "C". The challenge dose was 200 mouse LD<sub>50</sub>. Results are shown in Table 16. All mice which had received the highest dose (approximately 530 PFU) of SP-BKM 551 resisted challenge, while all but one of eight which received an estimated five PFU also were protected. This result indicates that multiplication of SP-BKM 551 occurred in newborn mice, causing no illness but providing protection against subsequent homotypic challenge.

A subsequent study has demonstrated that SP-BKM 551 virus can be isolated from the brains of suckling mice, beginning on the fourth day after IC inoculation and as late as the fourteenth day. Studies are continuing in an effort to determine the mechanism of interference observed with SP-BKM 551 and to obtain additional information on this apparently mouse-avirulent dengue plaque variant.

#### Laboratory Studies with Wesselsbron Virus

Wesselsbron virus has been isolated from Aedes mosquitoes at the Bang Phra study site, as reported in the SMRL Annual Reports for 1967 and 1968. Serologic evidence was obtained subsequently for infection in a variety of vertebrates, and a survey of school children showed age-specific antibody prevalence rates of 5-50% (also reported last year). In view of this evidence for human infection with Wesselsbron virus, a project has begun with the purpose of obtaining more information about this virus from both laboratory and field investigations. Thus far, only very preliminary studies have been done, consisting of a growth curve in monolayer cultures of LLC-MK2 cells and a measure of the pathogenicity of Wesselsbron virus administered by various routes in mice of various ages.

Table 17

Pathogenicity of Wesselsbron (BKM-367-66) virus in mice of various ages

Age of mice	Route of inoculation			
	intra cerebral	intra peritoneal	sub cutaneous	intra muscular
3 day	8.0 <sup>1</sup>	8.0	7.5	NT <sup>2</sup>
3 week	6.6	1.0	1.0	1.0
3 month	6.0	0.7	1.0	1.0

1. median lethal dose (log 10); volume IC = 0.02 ml; volume peripherally = 0.2 ml
2. not tested

Results of growth studies in LLC-MK2 cells indicated that a high multiplicity of infection (approximately 8 PFU/cell) resulted in maximal yield of virus in 48 hours, at which time supernatant fluid contained  $2.3 \times 10^4$  PFU/ml. A low multiplicity of infection resulted in maximal yields a day later (day 3) of  $2.1 \times 10^4$  PFU/ml. Titers of intracellular and extracellular virus were essentially equivalent and remained at the same levels for at least one week.

Studies in mice shown in Table 17, have indicated that Wesselsbron virus is pathogenic for 3 day old mice when inoculated either intracerebrally or peripherally. The virus is slightly less pathogenic via the IC route in 3 week old and 3 month old mice, while it fails to produce illness in these older mice when inoculated peripherally.

#### SUMMARY

Gibbons were used as a model for the study of sequential dengue infections with the four dengue serotypes; sequential heterotypic infections were demonstrated in spite of neutralizing and HI antibodies present at time of challenge. Infections were demonstrated by rise in antibody titer, and in many instances by detection of circulating virus.

An artificial feeding technique has been described for experimental dengue infection of Aedes mosquitoes, and some of the variables affecting such a system have been recognized. These include the passage history of the infecting strain, and the constituents of the suspending medium.

Plaque variants of dengue virus have been studied to determine antigenic composition and biologic significance of the plaque morphology. It appears that one such small plaque variant causes a non-lethal infection in suckling mice which protects mice against homotypic challenge infection.

Preliminary laboratory studies with Wesselsbron virus are described.