

Laboratory Study of Arboviruses

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OBJECTIVE: To investigate basic biological properties of the arboviruses, with particular reference to dengue viruses.

DESCRIPTION: Many strains of dengue 2 virus isolated from mosquito pools in 1967 epidemic of hemorrhagic fever at Koh Samui Island showed a mixture of small and large plaque size viruses on LLC-MK₂ cell culture. Pure cultures of small and large plaque viruses were prepared from strains BKM 551 in stock culture by methods as previously described. For the purpose of biological studies in various tests these two viruses were designated as SP-BKM 551 and LP-BKM 551 viruses. The passage level of MK₂ seed of SP-BKM 551 and LP-BKM 551 viruses used in these studies are the 13th and 14th.

PROGRESS: From the results of previous reports, SP-BKM 551 and LP-BKM 551 viruses were proved to be dengue 2 viruses but SP-BKM 551 was neutralized by monkey antiserum prepared from dengue 2 (NG-C) virus at a significantly less degree than LP-BKM 551. The other evidence that SP-BKM 551 and LP-BKM 551 are dengue 2 virus strains is that dengue 2 (NG-C) are neutralized to the same degree by monkey antisera immunized with SP-BKM 551 and LP-BKM 551 viruses.

One-day old mice inoculated with 5-530 PFU of SP-BKM 551 virus showed no sign or symptom but developed resistance to intracerebral challenge dose of 200-10,000 mouse LD₅₀ of dengue 2 (NG-C) virus 21 days after inoculation. Mice inoculated with 3-300 PFU of LP-BKM 551 virus showed sickness and death 8-10 days after inoculation.

In addition to the above studies small plaque size virus can be demonstrated in brains of infected mice after inoculation with SP-BKM 551 virus.

To exclude the contamination of dengue or other viruses in the seed of SP-BKM 551 virus, many attempts have been tried to illustrate SP-BKM 551 virus seed is a pure population.

1. After several passages in MK₂ cells there is stability of plaque size from passage 8 to passage 24.

2. LP-BKM 551 virus was introduced into SP-BKM 551 virus in different PFU ratios and the pools were inoculated intracerebrally into one-day old mice. The results shown in Table 1 illustrate when 0-30 PFU of LP-BKM 551 mixed with 240000 PFU of SP-BKM 551 deaths in mice occurred. None of the mice receiving 240000 PFU of SP-BKM 551 virus showed sickness or deaths.

3. From the results previously reported, three blind-passages of 1:10 dilution of 10% mouse brain suspension revealed no sickness or death in inoculated mice.

4. SP-BKM 551, LP-BKM 551 and BKM 551 (parent strain of SP and LP virus before cloning) were passed 4 times in one-day old mice, HA antigens prepared by the sucrose acetone method from infected mouse brain for each of the three viruses and tested against mouse immune ascetic fluids prepared against each of the four dengue serotype.

HI results are shown in Table 2, and indicate that all are identical to each other and to New Guinea C prototype.

When infected mouse brain suspensions of SP-BKM 551 virus, mouse passage 4, was tested with specific dengue monkey antisera types 1, 2, 3 and 4 by plaque reduction neutralization test, neutralization ratios were 15/640, 640/640, 10/80 and 15/400, respectively.

5. SP-BKM 551 virus from mouse passage 4 was used to immunize monkeys. Viruses were recovered from viremic monkey and reinoculated into another monkey. Virus was also recovered from the second monkey. Sera at two-month bleeding were tested for type-specific dengue antibodies. The results in Table 3 indicate SP-BKM 551 from 4th mouse passage. First monkey passage and second monkey passage showed the same neutralisation ratio when tested against dengue specific monkey antisera.

The first and second passage monkey isolates showed the same specific antibody response against dengue 2 virus as shown in Table 4.

Shifting of small plaque size to large plaque size.

Purification of small and large plaque virus for a pure clone was done with passage 8 of mixed-plaque BKM 551 virus. When the small plaque and large plaque were passed in MK₂ cells up to passage 24, the two viruses showed stability in plaque size, but there is evidence of shifting of small plaque size to large plaque size when the hosts were changed from MK₂ cells.

1. MK₂ seed of SP-BKM 551 virus was passed subcutaneously and intravenously into monkey and then the viruses recovered from viremic blood were passed into another monkey. After the first monkey passage, the virus recovered from viremic blood was large plaque variant. The virus recovered from viremic blood of the second monkey shows the same large plaque size as that of the first monkey.

2. SP-BKM551 virus was passed intracerebrally in one-day old mice with the appearance of large plaque size virus on mouse passage 4.

Changing of mouse virulence after monkey passage of SP-BKM 551.

There is an increase in mouse virulence after monkey passage of SP-BKM551 prepared from MK₂ cells. Two consecutive inoculations of monkeys by subcutaneous and intravenous route were done as described. In Table 5 the virus recovered from first viremic monkey showed mouse adaptation on second mouse passage, but at first only 1 of total 16 died on first mouse passage. When the first monkey virus was passed into another monkey, the virus recovered from viremic blood of the second monkey caused death in all inoculated mice at first mouse passage.

The studies of the challenge virus resistance in mice after IC inoculation of SP-BKM551 (MK₂-14).

Table 6 illustrates the results of challenge 21 days after IC inoculations of dilution of small plaque virus in newborn mice. Such mice resist IC challenge not only to dengue 2 New Guinea C, but also to chikungunya virus, a Group A arbovirus. While the protection endpoint was missed in this experiment, it can be seen

that protection against challenge virus corresponds fairly closely to the estimated intracerebral dose as expressed in terms of PFU, measured by a simultaneous titration of the inoculum in MK₂ cells. Serum antibody was measured in some of these mice at time of challenge, and the results are shown in Table 7.

While low levels of antibody could be detected against dengue 2 virus in mice, which received the largest inoculum of small plaque virus (4×10^5 PFU), no chikungunya antibody could be detected, nor could dengue antibody be measured in any of the mice which received higher dilutions of small plaque virus. Thus, it would seem that the protection of mice on day 21 is not the result of antibody against challenge virus. Virus content of brain tissue was measured during the same experiment on day 7, 14 and 21 following inoculation of small plaque virus, and the results are shown in Table 8.

At each interval, small plaque virus could be detected in brain tissue of mice which received doses ranging from 400,000 to 4 PFU, providing evidence for multiplication of the virus. It is clear from this table that titrations of brain tissue do not show a ten fold reduction in virus content corresponding to ten fold dilutions of the tissue, and this has been observed in several experiments. In fact, in some experiments very little or no virus can be detected in undiluted samples of brain tissue, suggesting that this virus demonstrates the phenomenon of autointerference.

It has been shown that no SP virus is detectable in the mouse brain after inoculation with a high dose (400,000, 40,000 and 4,000 PFU) but SP viruses persist in mouse brain inoculated with lower dose up to day 21 of inoculation.

The inoculated mice in every group inoculated with various doses from 4-400,000 PFU developed resistance to challenge virus so that simple persistence of SP virus in mouse brain is probably not the basis of protection.

Interferon studies in mouse brain inoculated with SP-BKM551 virus.

Interferon-like substance was prepared from mouse brain harvested at interval after inoculation of SP-BKM551 (MK₂-14), LP-BKM551 (MK₂-14) and dengue 2 (NG-C) Intracerebrally into one-day old mice. Twenty percent brain suspensions were centrifuged at 40,000 rpm for 4 hrs. The supernates were dialyzed in Hank's BSS at pH 2.0 for 12 hrs. and dialyzed back at pH 7.4 for 12 hrs. The preparations were found to be free of infectivity by plaque methods in MK₂ cell cultures. L cells were used in assay of mouse interferon.

To test interferon activity, 2.0 ml dilutions of each preparation were left for 18 hrs on cell sheets. After one washing with Hank's BSS, challenge viruses (Sindbis (cgLt 599) for L cells and Chikungunya (Ross) for MK₂ cells) were inoculated onto cell sheets in the dose of 30-100 PFU. Other plaque techniques followed the methods of dengue plaque on LLC-MK₂ cells.

The results illustrated in Table 9 reveal interferon action is probably not a mediator of mouse challenge virus resistance after SP-BKM551 (MK₂-14) inoculation. The peaks of virus titer, challenge virus resistance and interferon titer appeared on day 14. There was decrease in virus titer and interferon to undetectable levels by day 35.

The mouse challenge virus resistance on day 35 without detectable virus and interferon in brains suggests other mechanisms as another role in mouse immunity developed after SP-BKM551 (MK₂-14) inoculation. In Table 5 LP-BKM551 (MK₂-14) and dengue 2 (NG-C) showed interferon titers of 1:2560 and 1:10240. SP-BKM551 (MK₂-14), which caused no symptom in mice, produced interferon to a titer of only 1:80.

The interferon preparations of SP and dengue 2 (NG-C) showed no effect on MK₂ cells. Other physical properties of interferon of SP virus from mouse brain are being studied.

Studies of the production of interferon and virus titer in mouse brain at interval of time after high and low doses of SP-BKM551 on successive passages are in progress. The studies on mouse lethality of SP-BKM551 are one of the main interests of this investigation because of its apparent determinant of virulence.

Table 1. Results of Mouse Mortality from Inoculation of Pools of SP-BKM 551 and LP-BKM 551, LP-BKM 551 and SP-BKM 551 Viruses.

Dose of Pool of LP-BKM 551 and SP-BKM 551 (PFU)			Dose of LP-BKM 551 (PFU)			SP-BKM 551 (PFU)			
LP	SP	Mortality		LP	Mortality		SP		
3000	240000	8/8	8/8*	3000	8/8	8/8	240000	0/8	0/8
300	240000	8/8	8/8	300	8/8	8/8	240000	0/8	0/8
30	240000	7/8	8/8	30	6/8	6/8			
3	240000	2/8	2/8	3	6/8	3/8			
0	240000	1/8	0/8	0	2/8	2/8			

* Mortality ratio represents Deaths/Total Inoculated

Table 2. Hemagglutination-inhibition by DI-4 Immune Ascitic Fluids of D-2 (NG-C), BKM 551, LP-BKM 551 and SP-BKM 551 Antigens.

Prototype Immune Ascitic Fluid	Reciprocal HI Titer of Ascitic Fluid Against Indicated Antigen.			
	D-2 (NG-C)	BKM 551	LP-BKM 551	SP-BKM 551
D-1	80	320	80	160
D-2	1280	1280	1280	2560
D-3	160	160	160	160
D-4	80	320	80	320

Table 3. Plaque Reduction Neutralization of Virus Recovered from 2 Monkey-passages of SP-BKM 551 of Fourth Mouse-passage.

Virus from Monkey Passage	Antisera			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Passage 1	<10	140	<10	<10
Passage 2	10	640	<10	<10
Homologous Titer	640	640	80	400

Table 4. Antibody Response of Monkeys Inoculated with Mouse Passage 4 of SP—BKM 551 Virus

Monkey Passage Level	Reciprocal 50% Plaque Reduction Against Indicated Virus			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Passage 1	80	> 640	10	60
Passage 2	30	550	> 10	45
Homologous titer	640	640	80	400

Table 5. Mouse Pathogenicity of SP—BKM 551 after Two Passages in Monkeys

Monkey Passage	Mouse Passage (Intracerebral)					
	First		Second		Third	
	Mortality	Virus Titer (PFU/0.3 gm.) of mouse brain	Mortality	Virus Titer (PFU/0.3 gm.) of mouse brain	Mortality	Virus Titer (PFU/0.3 gm.) of mouse brain
(800) Before Monkey Passage	0/8*	$4.0 \times 10^{2.7}$	0/8	$2.3 \times 10^{5.7}$	0/8	$5 \times 10^{5.7}$
(36) First Passage	0/8 1/8	$1.1 \times 10^{5.3}$	8/8 8/8	$1.9 \times 10^{5.2}$	8/8 8/8	$4.6 \times 10^{7.3}$
(60) Second Passage	8/8 8/8	$1.3 \times 10^{5.3}$	8/8 8/8	$2.0 \times 10^{7.3}$	8/8 8/8	$2.0 \times 10^{7.3}$

*Mortality ratio represents deaths/Total Inoculated

() Indicates PFU dose of mouse intracerebral inoculation

Table 6. Challenge Virus Resistance in Mice 22 Days after IC Inoculation of BKM 551—SP (MK 2—14)

Inoculum Dilution of (BKM 551—SP)	Estimated IC Dose (PFU/0.3 ML)	Mortality		
		21 Days After BKM 551—SP Inoc.	11 Days after Challenge with $\leq 10,000$ Mouse LD of	
			D—2 (NG—C)	Chikungunya
Undiluted	400,000	0/24*	0/8	0/8
—1	40,000	0/24	0/8	0/8
—2	4,000	0/24	0/8	0/8
—3	400	0/24	0/8	0/8
—4	40	0/24	0/8	0/8
—5	4	0/24	2/8	0/8

*Number dead/Number inoculated

Table 7. Antibody Response 21 Days After Inoculation of BKM 551—SP (MK 2—14) in Newborn Mice

Inoculum (Dilution of BKM 551—SP)	Estimated IC Dose (PFU/0.03 ML)	Reciprocal 50% Plaque Reduction Titer Against Indicated Virus	
		D—2 (NG—C)	Chikungunya
Undil	400,000	40	<10
—1	40,000	20	<10
—2	4,000	<10	<10
—3	400	<10	<10
—4	40	<10	<10
—5	4	<10	<10

Table 8. Growth of BKM 551-SP (MK 2-14) in CNS of Newborn Mice

Inoculum (Dilution of (BKM 551-SP))	Estimated IC Dose (PFU/0.03 ML)	Virus Content (PFU/0.3 ML)* of Brain Tissue on Indicated Day of Infection											
		Day 7			Day 14			Day 21					
		Undil.	-1	-2	-3	Undil.	-1	-2	-3	Undil.	-1	-2	-3
UNDIL.	400,000	TNTC	74	42	9	69	43	67	33	0	0	0	0
-1	40,000	70	36	65	6	50	36	53	18	0	0	0	0
-2	4,000	100	17	18	2	17	5	2	1	0	0	0	0
-3	400	88	38	13	3	67	52	72	29	25	22	8	5
-4	40	25	14	3	0	113	56	19	9	25	14	5	2
-5	4	28	15	3	0	120	33	31	5	TNTC	TNTC	60	16

*Expressed as Mean Count of Triplicate Bottle Cultures

Table 9. Correlation of Virus Titer, Challenge Virus Resistance and Interferon Titer in One-day Old Mice Inoculated Intracerebrally with SP-BKM 551 (MK 2-14)

Day of Inoculation	Virus Titer PFU/0.3 gm brain	Day after Challenge with $\geq 10,000$ mouse LD ₅₀ of chikungunya virus	Interferon in 2.0 ml. of 10% mouse brain
1	0	8/8*	<10**
2	$10^{3.0}$	8/8	<10
3	$2.2 \times 10^{3.0}$	8/8	<10
4	$2 \times 10^{3.0}$	8/8	<10
5	$1.7 \times 10^{4.0}$	7/8	<10
6	$1.4 \times 10^{4.0}$	3/8	<10
7	$3.2 \times 10^{4.0}$	2/8	20
14	$1.0 \times 10^{6.7}$	0/8	80
21	$1.0 \times 10^{5.7}$	0/8	20
28	$0.6 \times 10^{1.0}$	0/8	0
35	0	0/8	0

* Mortality ratio=Deaths/Total inoculated

** Reciprocal 50% plaque reduction of interferon dilutions to Cglt 599

Table 10. Effect of Interferon Preparation from SP-BKM 551, LP-BKM 551 and Dengue 2 (NG-C) to L Cells and MK₂ Cells

Viruses	Reciprocal 50% Plaque Reduction of Interferon Dilution	
	L Cells*	MK ₂ Cells**
SP-BKM 551 (MK ₂ - 14)	80	<10
LP-BKM 551 (MK ₂ - 14)	2560	—
Dengue 2 (NG-C)	10240	<1:20

* Challenge virus after interferon P_x = Cglt 599

** Challenge virus after interferon P_x = Chikungunya (Ross)