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KUROTORI VIRUS: A VIRUS NEWLY ISOLATED FROM *ANOPHELES SINENSIS* AND PATHOGENIC TO MICE.

I. ISOLATION AND PROPERTIES

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SUMMARY A virus which is pathogenic to mice was isolated from the mosquito, *Anopheles sinensis*, captured at Kurotori-cho (latitude 34.5 N; longitude 135.5 E) in Osaka in 1963. The agent multiplied well in BHK-21 cells producing severe cytopathic effects. The virus was sensitive to ethylether, sodium deoxycholate (SDC) and to pH values of 5.0 or less, but 5-iododeoxyuridine (IUDR) did not inhibit its multiplication, suggesting that it contains ribonucleic acid as genetic material. Its particle size was estimated by filtration to be about 50 mμ. Complement fixing antigens were obtained from the brains of infected suckling mice, but hemagglutinins to red blood cells of one-day-old chicks were not extracted from infected mouse brains. From these characteristics, this virus, Kurotori virus, was classified in the Arbovirus group.

INTRODUCTION

Since 1963, many studies have been made in our laboratory on the ecological features of Japanese encephalitis virus (JEV).

During this period, we isolated 7 arboviruses besides JEV and all but one of the viruses isolated in 1963 were grouped as A arboviruses belonging to the Getah complex. (Scherer et al, 1962).

The exceptional virus was isolated from *Anopheles sinensis* and was found not to belong to either group A or B of the arboviruses.

No one in Japan, has previously isolated any virus, including JEV, from *Anopheles sinensis*.

MATERIALS AND METHODS

1. *Collection of mosquitoes*

Mosquitoes were collected for one hr just after sunset by aspiration of using a net or dry ice trap.

2. *Isolation of virus*

On Sept. 16, 1963 four female *Anopheles sinensis* mosquitoes were captured. These mosquitoes were fed and kept overnight at room temperature and then preserved at −70 C until examined. Several

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mosquitoes were homogenized together in a precooled mortar with 6.0 ml of phosphate-buffered saline, pH 7.0, supplemented with 5% JEV-antibody-free young chicken serum, penicillin (500 IU/ml) and streptmycin (500 μg/ml). The homogenate was centrifuged at 10,000 rpm for 10 min at 0 C and the supernatant was inoculated into suckling mice intracerebrally (0.02 ml) or intraperitoneally (0.1 ml). The mice were observed for 14 days after inoculation. Mice showing pathological symptoms were killed and their brains were homogenized in the buffer described above to make 10% homogenates. The homogenates were centrifuged, diluted 1:100 and inoculated into suckling mice intracerebrally. After the second intracerebral passage, the brains of mice showing symptoms were used for virological tests.

3. *Animals*

ddY Mice were used throughout. For isolation of virus, suckling mice were used within 4 days after birth. Rabbits and guinea pigs were purchased from local dealers.

4. *Tissue culture cells*

For primary tissue culture, chick embryo cells, cynomolgus monkey kidney cells and rabbit kidney cells were used. The established cell lines used were BHK-21, HeLa and GMK (a cell line of African green monkey kidney cells established by Dr. Nakano, NIH, Japan).

The medium used for tissue culture was LE (0.5% lactalbumin hydrolysate in Earle's balanced salt solution) or YLE (0.1% yeast extract, 0.5% lactalbumin hydrolysate in Earle's balanced salt solution) medium supplemented with 10% calf serum. After inoculation of virus, medium containing 2–5% calf serum (free of anti-JEV antibody) was used.

5. *Serological tests and preparation of antigen*

The serological tests employed were the complement fixation (CF) test and neutralization (NT) test using standard tube methods (Lennette and Schmitt, 1964). Infected mouse brain at the second in vivo passage level was used for preparation of antigens by extraction using acetone-ether or sucrose acetone (Clarke and Casals, 1958). This antigen preparation was used for the hemagglutination (HA) test or CF test and preparations were diluted with borate buffer, pH 9.0 supplemented with 0.4% egg albumin and with veronal buffered saline, pH 7.2, in the HA test and CF test, respectively (Clarke and Casals, 1958).

6. *Virus*

The Nakayama-NIH and JaGAr 01 strains of JEV, Getah (AMM2021) of group A arbovirus, Theiler's virus, polio virus type 1, Mahoney strain, and herpes simplex virus type 1 were used.

7. *Estimation of sensitivity of virus to chemicals*

Virus was treated with ethylether, sodium deoxycholate (SDC), protamine or $MgCl_2$ (Andrews and Horstman, 1949; Theiler, 1957; Wallis and Melnick, 1962), and then inoculated into suckling mice intracerebrally and its effect was compared with that of untreated, control virus.

Viral nucleic acids were determined using 5-iodo-2′-deoxyuridine (IUDR). This drug was added to the maintenance medium at a final concentration of 10^{-5} M. Titrations of the virus were performed in the presence and absence of IUDR (Justines and Kuns, 1970). Herpes simplex virus and JEV were used as control DNA and RNA type viruses, respectively.

8. *pH Stability*

A series of solutions of 0.01 M disodium phosphate-citric acid buffer of pH 3.0 to 8.0 was prepared. Ten per cent homogenates of infected mouse brain in physiological saline were mixed with an equal volume of each buffer solution. The mixtures were kept at 37 C for 1 hr and then the pH of each was determined. Serial 10-fold dilutions of each mixture were assayed for infectious virus.

9. *Estimation of size of infective agent*

Infected suckling mouse brains were homogenized in a mortar in bovine-albumin phosphate buffered saline and diluted to give 5 per cent suspensions. The suspensions were centrifuged at 10,000 rev/min. for 30 min. in a refrigerated centrifuge. The supernatant fluid was filtered successively through Millipore membranes of 450, 220, 100 and 50 mμ pore size, using a syringe-type apparatus. Serial 10-fold dilutions of each filtrate were inoculated into tissue culture cells and the titer of each sample was calculated by the 50% end point method (Reed and Muench, 1938). The JaGAr 01 strain of JEV and AMM2021 strain of Getah virus were used as control viruses to confirm the filtrability of virus through Millipore membranes.

RESULTS

1. *Process of isolation*

The procedure for isolation of Kurotori virus is shown in Fig. 1. Aliquots of the inoculated specimens were tested for bacteria and fungi but results were negative. It is evident that the original mosquitoes had some agent which was pathogenic to mice and this agent seemed to be a virus.

The virus was named Kurotori virus after the name of the place where the mosquitoes were captured.

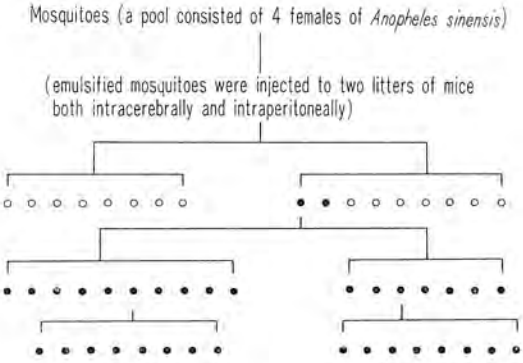

FIGURE 1. *Isolation of Kurotori virus.* ●, *mouse showed typical encephalitic symptoms and died;* ○, *mouse showed no symptoms for 14 days after inoculation.*

2. *Characteristics of antigen extracted by acetone-ether*

The antigen extracted with acetone-ether did not show HA activity with one-day-old chick red blood cells at pH 6.0 to 7.0. As shown in Table 1, there is no cross reaction between Kurotori virus and JEV (Nakayama-NIH strain) or Theiler's virus, which are both known to cause paralysis of mice.

3. *Infectivity of Kurotori virus to animals and tissue culture cells*

Since it was necessary to distinguish newly isolated virus from other known viruses, the sensitivities of various animals and tissue culture cells to this virus were studied to develop an adequate system for a neutralization test.

Suckling mice were very sensitive to the virus and showed severe symptoms of encephalitis after its intracerebral or intraperitoneal inoculation (Fig. 2). The latent period of the disease varied with the route of inoculation, the dose and the age of the mice, being 2 to 6 days. Adult mice were resistant to intraperitoneal inoculation of Kurotori virus but were as sensitive as suckling mice to intracranial challenge. Rabbits were insensitive to the virus, even on intracranial challenge.

FIGURE 2. *Suckling mice showing typical encephalitic symptoms on the 6th day after inoculation.*

Two established cell lines, GMK and BHK-21 showed cytopathogenic effects (CPE) after inoculation of the virus. BHK-21 cells showed the greatest CPE (Fig. 3) and had the highest sensitivity to the virus (Table 2).

In following experiments virus titration and neutralization of virus were done with BHK-21 cells or suckling mice.

TABLE 1. *Complement Fixation reactions between several viruses and antisera against them*

Serum	Antigen	JEV (Nakayama-NIH)							Kurotori virus							Theiler's virus							Normal					
		4	8	16	32	64	128	256	4	8	16	32	64	128	256	4	8	16	32	64	128	256	4	8	16	32	64	128
JEV (Nakayama-NIH)	4	4	4	4	4	4	4	0	1	1	0	1	1	0	0	2	2	2	1	1	1	0	0	2	1	1	0	0
	8	4	4	4	4	4	4	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	16	4	4	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	32	4	4	4	4	4	3	0	0	0	0	0	0	0	0													
	64	4	4	4	4	4	2	0																				
	128	1	1	4	4	3	1	0																				
	256	0	1	3	2	1	1	0																				
	512	0	0	1	1	0	0	0																				
Kurotori virus	4	2	1	1	1	1	0	0	4	4	4	2	1	0	0	2	2	1	1	0	0	0	1	1	0	0	0	0
	8	0	0	0	0	1	0	0	4	4	4	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	4	4	4	2	0	0	0	0	0	0	0	0	0	0						
	32								4	4	4	2	0	0	0													
	64								4	4	4	2	0	0	0													
	128								4	4	3	2	0	0	0													
	256								4	3	2	1	0	0	0													
	512								3	1	1	1	0	0	0													
	1024								0	0	0	0	0	0	0													
Normal	4	2	2	2	2	1	1	1	0	0	0	0	0	0	0	2	2	2	2	1	1	1	2	2	2	2	1	0
	8	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 2. *Titration of Kurotori virus in mice, rabbits and different tissue culture cells*

A) Animals (log LD_{50}/ml)

Mice					Rabbits
Suckling			Adult		
intracerebral inoculation	intraperitoneal inoculation	subcutaneous inoculation	intracerebral inoculation	intraperitoneal inoculation	intracerebral inoculation
9.1	7.0	6.3	8.1	2.2	≤ 1.2

B) Tissue Culture Cells (log $TCID_{50}$/0.2 ml)

Cell line			Primary cells		
GMK	BHK-21	HeLa	Chick embryo	Monkey kidney	Rabbit kidney
5.5	8.3	3.5	5.5	≤ 0.5	5.3

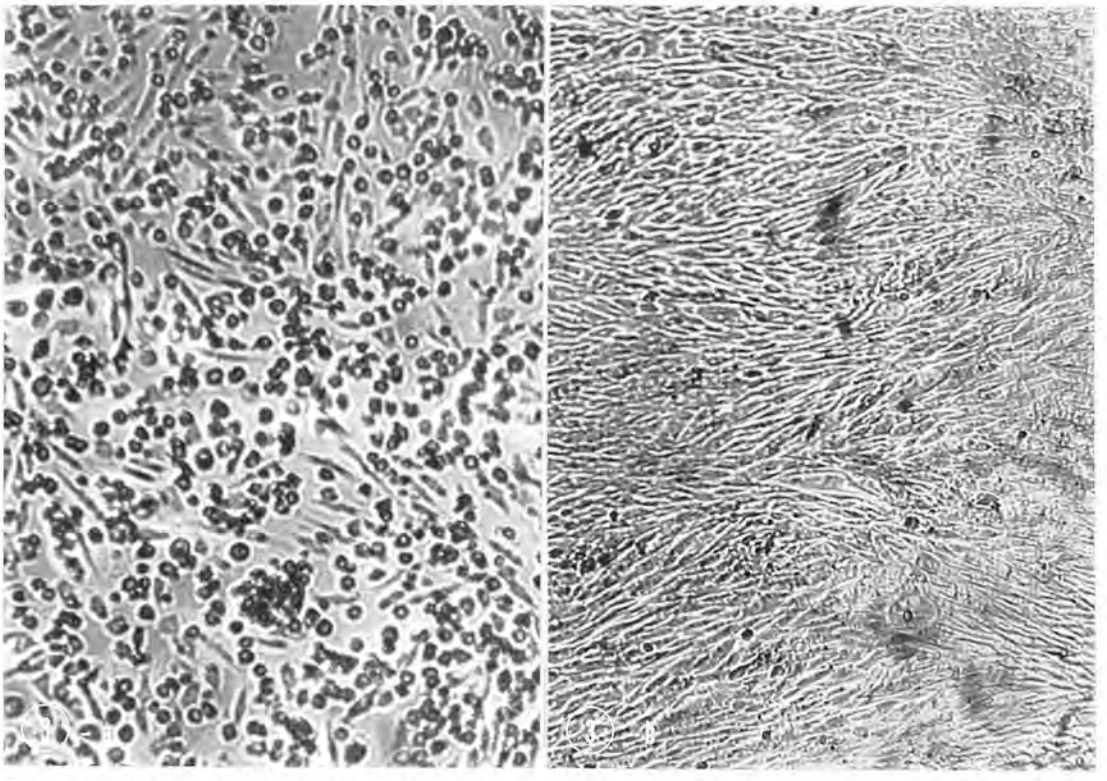

FIGURE 3. *Cytopathic effect (CPE) of Kurotori virus in BHK-21 cells. CPE of the virus in a monolayer 6 days after infection (a) and uninfected control monolayer (b).*

4. *Neutralization test*

To clarify the antigenic relationship between Kurotori virus and two other arboviruses isolated in Osaka prefecture, neutralization tests were carried out in a tissue culture system using Kurotori virus, JEV and Getah virus and hyperimmunized mouse sera against them. High antibody titers were detected in homologous systems but no cross reactions were observed.

5. *Size of Kurotori virus particles*

As shown in Table 3, the size of Kurotori virus was estimated by comparison with those of Getah virus and JEV. It was concluded that Kurotori virus was about as large as the other two virus strains, that is about 50 mμ or less in size.

TABLE 3. *Estimation of particle size of Kurotori virus by filtration*[a]

Virus	Exp. No.	Pore size (mμ)				Not filtered
		50	100	220	450	
Kurotori virus	1	4.5	—	5.9	6.7	6.7
	2	4.0	—	5.5	6.0	7.0
	3	6.0	6.5	6.5	6.75	6.25
Getah virus	1	6.5	5.1	7.3	7.9	8.1
	2	6.9	5.7	7.9	7.9	8.3
JEV (Nakayama)	1	4.5	3.5	6.5	6.5	7.5

a Titers are expressed as log $TCID_{50}$/0.2 ml.

6. *Effects of chemicals on viral infectivity*

Sensitivity to ethylether or SDC is an important character in identification of Arboviruses. Table 4 shows results on the sensitivities of Kurotori virus to these compounds and to protamine and $MgCl_2$. Although Kurotori virus was resistant to ether and SDC at the 3rd in vivo passage, it became sensitive to them after the 8th in vivo passage. The sensitiveness of Kurotori virus to Mg^{++} excludes the possibility that it belongs to the enterovirus group.

7. *Effect of 5-iodo-2′-deoxyuridine on growth of Kurotori virus in BHK-21 cells*

To identify the genetic material of Kurotori virus, BHK-21 cells were treated with 5-iodo-2′-deoxyuridine and then infected with Kurotori virus, JEV (JaGAr 01) or Herpes simplex virus (type 1). Unlike HSV, the

TABLE 5. *Effect of 5-iodo-2′-deoxyuridine on growth of Kurotori virus*[a]

Virus	5-iodo-2′-dooxyuridine	
	Treated	Control
Kurotori virus	8.1	7.5
JEV (JaGAr 01)	5.9	6.7
Herpes simplex virus (type 1)	0	3.5

a Titers are expressed as log $TCID_{50}$/0.2 ml.

TABLE 4. *Effects of chemicals on the injectivity of Kurotori virus*

Virus		Ether		SDC		Protamine		Mg^{++}	
		treated	control	treated	control	treated	control	treated	control
Kurotori virus[a]	P. 3	3.8	4.8	3.8	4.8	4.6	5.5	2.8	6.4
	P. 8	<2.0	6.5	0.8	6.7				
JEV (JaGAr 01)[a]		3.4	7.9	0.7	7.5				
Polio virus (type 1)		6.5[b]	6.5[b]						

a Titers are expressed as log LD_{50}/0.025 ml.
b Titers are expressed as log $TCID_{50}$/0.2 ml in HeLa cells.

growth of Kurotori virus was not inhibited by the drug (Table 5).

8. *pH Stability*

The stability of the virus at various pH values is shown in Fig. 4. The infectivity of the virus was stable at pH 6.0, 7.0 and 8.0, but it was completely lost at pH 4.0 or 3.0. The pH stability of the virus is quite similar to that of JEV (JaGAr 01), but polio virus is stable over a wide pH range.

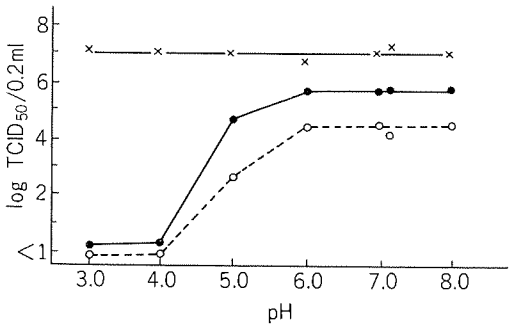

FIGURE 4. *Effect of pH on stability of Kurotori virus.* ×——×, *polio;* ●——●, *Kurotori;* ○- - -○, *JaGAr 01.*

DISCUSSION

In Osaka, *Anopheles sinensis* is the second commonest mosquito after *Culex*. Various virus strains, such as Anopheles A and Anopheles B, have been isolated from *Anopheles* in Colombia (Roca-Garcia, M, 1944) but this is the first time virus has been isolated from *Anopheles* in Japan. The relationship between the viruses isolated in Colombia and Kurotori virus is unknown. The most possible contaminant viruses to be isolated from mice in our laboratory are Theiler's virus, lymphocytic choriomeningitis (LCM) virus and JEV. We checked other mice in our vivarium for LCM at the time of isolation of Kurotori virus but could find no sign of their infection with it. The results in Table 1 show that Kurotori virus is quite different from the other two possible contaminant viruses, JEV and Theiler's virus. As already stated in the introduction, six other viruses, other than JEV, which we isolated from mosquitoes all belonged to the Getah complex. Kurotori virus does not cross-react with anti-Getah virus serum.

Filtration experiments showed that Kurotori virus particles are less than 50 mμ in size and this is within the range of size of arboviruses. Although the infectivity of Kurotori virus was resistant to ether or SDC treatment at the 3rd in vivo passage, it became sensitive to these chemicals at the 8th in vivo passage. A similar phenomenon has been observed with other newly isolated arboviruses. The results of 5-iodo-2′-deoxyuridine treatment indicate that the genetic material of Kurotori virus is RNA. From these results it is concluded that Kurotori virus is an arbovirus but does not belong to group A or B.

Our main interests in Kurotori virus are its relationship to human encephalitis of unknown etiology and its ecological characteristics.

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