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GROWTH OF JAPANESE ENCEPHALITIS VIRUS IN ESTABLISHED LINES OF MOSQUITO CELLS

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SUMMARY Growth of Japanese encephalitis virus was studied in three kinds of mosquito cell lines, that is, Singh's *Aedes albopictus* (SA), Singh's *A. aegypti* (SE) and Peleg's *A. aegypti* (PE) cells. The virus yield at 28 C was highest in SA, followed by that in PE and then that in SE cells. Viruses with passage histories in MK or BHK21 cells seem to produce higher titers of virus in SA cells than those which had been passaged in suckling mouse brain, although the latter can grow well in BHK21 cells at 37 C. Using JaOH-0566 strain which had been passaged in MK and BHK21 cells, the highest yield of the virus, more than 10^9 PFU/ml, was obtained in SA cells. Active growth of the virus in SA cells was observed between 22 and 37 C, although the growth rate was very slow at 22 C. Steady growth of the virus was not observed at temperatures below 15 C or above 40 C.

INTRODUCTION

Much less is known about the growth of arboviruses in poikilothermic animal cells, including arthropod cells, than in mammalian or avian cells. One merit of using such cells is that growth of viruses can be studied at lower temperatures than those used for mammalian or avian cells. As the first step toward this, it is necessary to find an appropriate system to get a high titer of the virus. Accordingly we studied the growth of Japanese encephalitis virus (JEV) in the established lines of mosquito cells now available, that is, Singh's *Aedes albopictus* (SA) and *A. aegypti* (SE) cells (Singh, 1967), and Peleg's *A. aegypti* (PE) cells (Peleg, 1968b).

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MATERIALS AND METHODS

1. *Japanese encephalitis virus (JEV)*

The Nakayama strain, which had been passaged more than 26 times in adult mouse brain (MB) and 6 times in suckling mouse brain (SMB), the JaGAr-01 strain, at the 9th passage in SMB, and the JaOH-0566 strain at the second passage in SMB were supplied by Drs. T. Fukunaga and N. Ueba of the Public Health Institute of Osaka Prefecture. Infected SMB were homogenized with PBS (Dulbecco and Vogt, 1954) to make 10% homogenates and centrifuged at $1,500 \times g$ for 15 min, and the supernatants were stored at −70 C. Before use for inoculation,

these specimens were diluted with 0.2% gelatin in YLE (0.5% lactalbumin hydrolysate, 0.1% yeast extract in Earle's balanced salt solution). The JaOH-0566 strain, which had been passaged three times in SMB and 62 times in primary cultures of monkey kidney (MK) cells was supplied by Dr. I. Yoshida of the Kannonji Institute, The Research Foundation for Microbial Diseases of Osaka University. In this laboratory, these strains were passaged in BHK21 cell monolayers several times with fluid medium consisting of 2% calf serum in Eagle's minimal essential medium (MEM, Eagle, 1959), purified three times by plaque isolation on BHK21 cells and multiplied in BHK21 cells in fluid medium.

2. *Cells*

Established lines of Singh's *A. albopictus* (SA), and *A. aegypti* (SE), and Peleg's *A. aegypti* (PE) cells (Singh, 1967; Peleg, 1968b) were kindly supplied by Dr. S. Makino, Department of Microbiology, Kobe University. These cells were grown as monolayers in rubber-stoppered prescription bottles at 28 C, with a 1 : 1 mixture of MEM and Mitsuhashi-Maramorosch's (MM) medium (Mitsuhashi and Maramorosch, 1963) supplemented with 10% calf serum. An established line of baby-hamster kidney-cells, BHK21 clone 13 (MacPherson and Stoker, 1962) was obtained from Dr. S. Hotta, Department of Microbiology, Kobe University, and was grown at 37 C in MEM supplemented with 10% bovine serum.

3. *Inoculation and growth of JEV*

The growth medium was removed from confluent sheets of cultured cells in 2 ounce bottles, and the cell sheets were washed twice with 4 ml of PBS. Each sheet was inoculated with 0.2 ml of seed virus. Then the sheets were incubated for 2 hr, at 28 C (for mosquito cells) or 37 C (for BHK21 cells), spreading the virus every 30 min to allow adsorption. Then the cell sheets were washed twice with 4 ml of PBS to remove unadsorbed virus, and 3 ml of virus phase medium (1 : 1 mixture of MM and MEM supplemented with 2% calf serum free of JEV antibody) were added. After an appropriate time of incubation at 28 C (for mosquito cells, except in the experiment of Fig. 2) or 37 C (for BHK21 cells), portions of the virus phase medium from replicate cultures were taken out, pooled and assayed for JEV. To measure cell-associated virus, infected cell sheets of replicate cultures were scraped into PBS and homogenized in a Teflon homogenizor and then assayed for JEV.

4. *Hemagglutination (HA) of JEV*

A microtiter method (Sever, 1962) was applied using the procedure of Clarke and Casals (1958) with goose red blood cells suspended in VAD 6.2.

5. *Infectivity titration of JEV*

Specimens were serially diluted 10 fold with 0.2 % gelatin in YLE medium. Growth medium was removed from confluent sheets of BHK21 cells in 2 ounce bottles. Cell sheets were washed twice with 4 ml of PBS, and inoculated with 0.2 ml of diluted virus specimen. Adsorption was carried out at 37 C for 2 hr, spreading the virus every 30 min. The first overlay medium was 1% agar in YLE supplemented with 2% calf serum free of JEV antibody. After solidification of the agar, bottles were inverted and incubated at 37 C for 3 days. Then a second overlay medium, with the same constitution as the first but containing 0.01% neutral red was added. Plaques were counted on the 4th day and titers were recorded as plaque forming units (PFU) per ml.

RESULTS

1. *Yields of JEV in mosquito cells*

Three strains of JEV with different passage histories were tested for their capacity to produce progeny viruses in 3 established lines of mosquito cells in 5 days at 28 C. The virus yield at 37 C in BHK21 cells was used as a control. Results are summarized in Fig. 1 and Table 1.

SA cells gave the highest titer of JEV, followed by PE, while SE gave the lowest. The ratios of PFU to HA of the viruses grown in SA were 10^5–10^7. Viruses passaged in MK or BHK21 cells seemed to give a higher titer in SA cells than those passaged in SMB only, although the latter gave a high titer in BHK21 cells. Viruses which had been passaged in MK or BHK cells gave a higher maximum titer in SA cells than in BHK21 cells, and the reverse was true for those passaged in SMB. When the latter viruses were propagated once

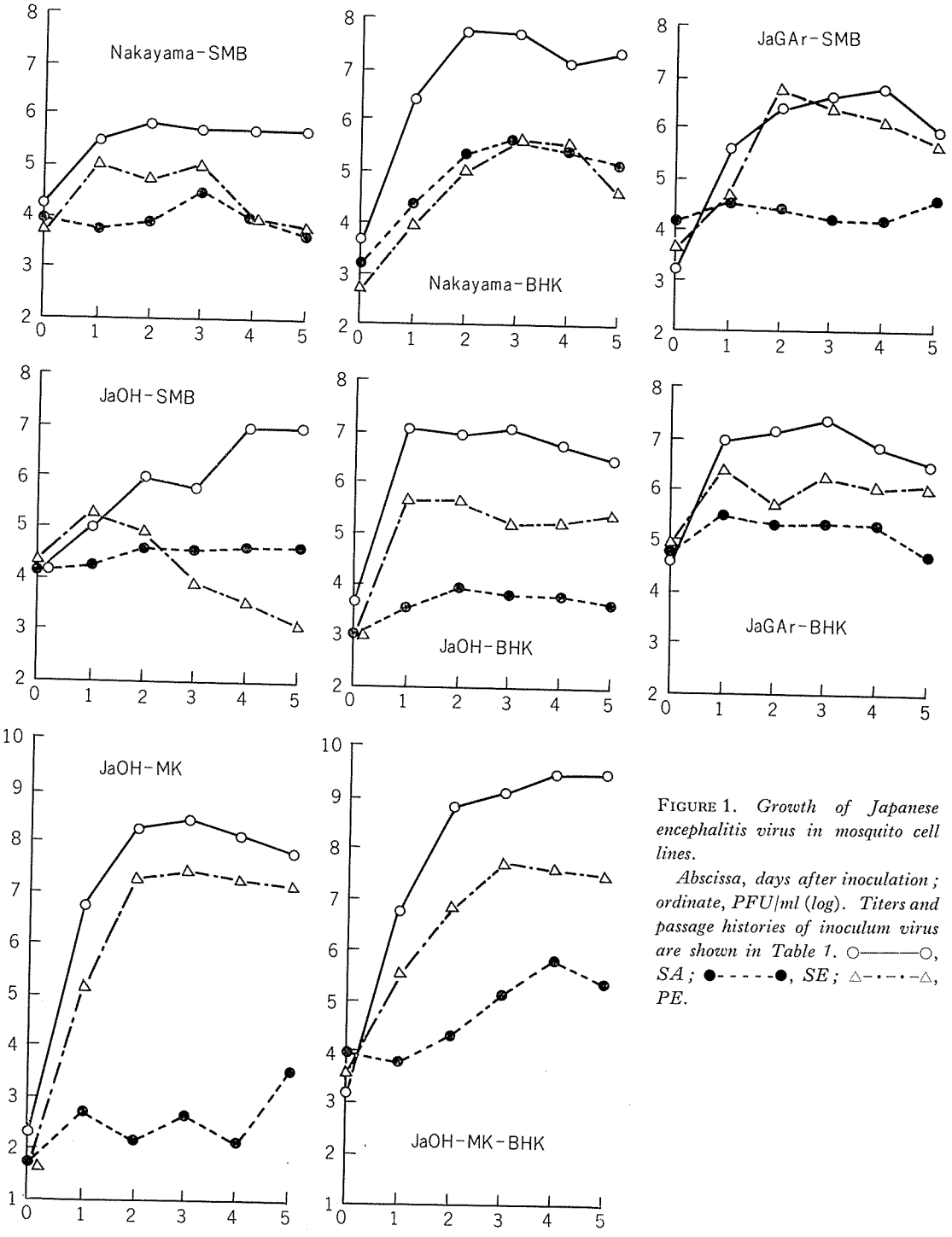

FIGURE 1. *Growth of Japanese encephalitis virus in mosquito cell lines.*

Abscissa, days after inoculation; ordinate, PFU/ml (log). Titers and passage histories of inoculum virus are shown in Table 1. ○———○, *SA;* ●- - - -●, *SE;* △-·-·-△, *PE.*

TABLE 1. *Maximum titers of JEV grown in mosquito cell lines and in BHK21 cells*

Seed virus inoculated			Maximum titer[a]			
Strain	Passage history[b]	Titer[a]	SA	SE	PE	BHK
Nakayama	$MB^{26}SMB^{6}$	7.6	5.8 (2)	4.5 (3)	5.0 (3)	7.6 (1)
	$MB^{26}SMB^{6}BHK^{8}$	6.3	7.6 (2)	5.6 (3)	5.6 (3)	7.0 (2)
JaGAr-01	SMB^{9}	7.3	6.7 (4)	4.6 (5)	6.7 (2)	7.4 (1)
	$SMB^{9}BHK^{11}$	7.7	7.4 (3)	5.5 (2)	6.4 (1)	7.2 (1)
JaOH-0566	SMB^{2}	8.0	6.9 (4)	4.7 (4)	5.4 (1)	7.5 (1)
	$SMB^{2}BHK^{11}$	6.4	7.0 (3)	4.0 (2)	5.6 (2)	7.0 (2)
	$SMB^{3}MK^{62}$	5.3	8.3 (3)	3.5 (5)	7.4 (3)	7.9 (1)
	$SMB^{3}MK^{62}BHK^{14}$	7.1	9.4 (4)	5.8 (4)	7.7 (3)	8.7 (2)

[a] Titers are expressed as PFU/ml (log). Numbers in parentheses are days after infection when the maximum titer of JEV was observed.

[b] MB, adult mouse brain; SMB, suckling mouse brain; Passages in BHK include three plaque-isolations.

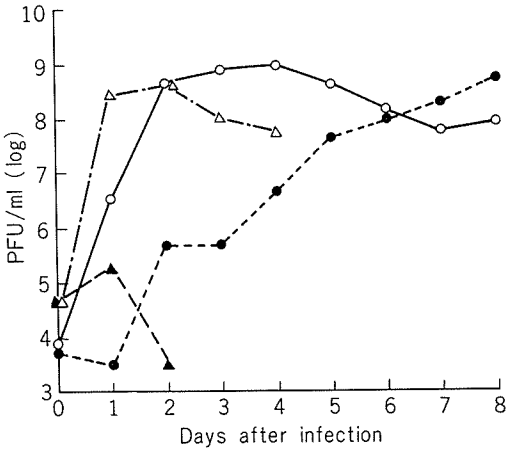

FIGURE 2. *Growth of JEV, JaOH-0566 strain, in Singh's Aedes albopictus cells at different temperatures.* ●- - - -●, 22 *C*; ○———○, 28 *C*; △-·-·-△, 37 *C*; ▲-----▲, 40 *C*.

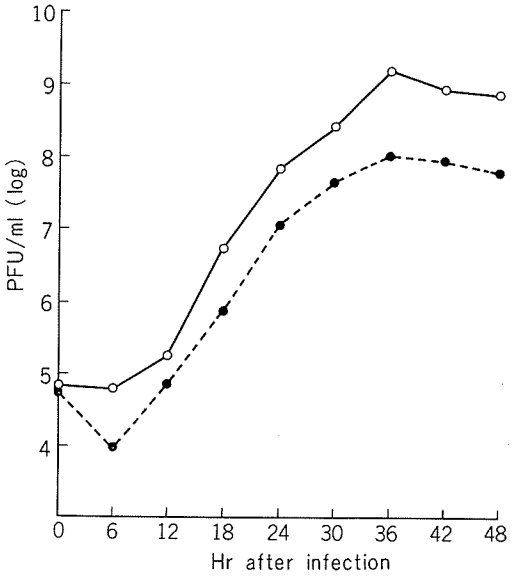

FIGURE 3. *Growth of JEV, JaOH-0566 strain, in Singh's Aedes albopictus cells at* 28 *C.* ○———○, *Fluid virus;* ●- - - -●, *cell-associated virus.*

or twice in BHK21 cells, the progenies seemed to grow to a higher titer in SA cells than in BHK21 cells and this tendency did not change after plaque isolations (Table 2).

2. *Growth of JEV, JaOH-0566 strain in SA cells*

Subsequently, we used the JaOH-0566 strain which had been passaged in MK and BHK as seed virus, because this virus produced the highest yield in SA cells, more than 10^9 PFU/ml. Infected SA cells were incubated at various temperatures from 4 to 40 C, and the virus titer was measured every day. Active

TABLE 2. *Maximum titers of JEV grown in BHK21 and Singh's Aedes albopictus cells*

Strain, history before BHK21 passage[b]	Passages in BHK21 cells[c]	Titer[a] of seed virus	Maximum titer[a] in	
			SA	BHK21
Nakayama, $MB^{26}SMB^{6}$	0	7.7	7.3 (1)	7.6 (1)
	PB	6.2	7.3 (2)	7.0 (1)
	$(PB)^{2}$	6.0	8.2 (3)	7.1 (2)
	$(PB)^{3}$	6.0	7.0 (2)	7.0 (1)
	$(PB)^{3}B$	6.3	7.5 (3)	7.1 (1)
	$(PB)^{3}B^{2}$	6.4	7.8 (3)	7.0 (2)
JaGAr-01, SMB^{9}	0	7.5	7.0 (3)	7.4 (1)
	B	4.7	8.3 (3)	6.3 (2)
	B^{2}	4.8	8.0 (3)	7.2 (2)
	B^{3}	7.2	7.4 (2)	7.6 (1)
	$B^{3}PB$	6.0	7.9 (2)	6.8 (2)
	$B^{3}(PB)^{2}$	6.3	8.0 (3)	7.5 (2)
	$B^{3}(PB)^{3}$	5.5	7.8 (3)	5.4 (2)
	$B^{3}(PB)^{3}B$	6.9	8.2 (2)	7.2 (1)
	$B^{3}(PB)^{3}B^{2}$	7.5	8.3 (2)	7.3 (1)
JaOH-0566, SMB^{2}	0	8.0	6.9 (4)	7.5 (1)
	B	7.5	5.4 (3)	6.7 (1)
	B^{2}	6.5	6.6 (2)	6.2 (1)
	B^{3}	5.1	7.2 (3)	7.2 (2)
	$B^{3}PB$	5.6	7.6 (3)	5.2 (2)
	$B^{3}(PB)^{2}$	5.7	7.7 (3)	5.4 (2)
	$B^{3}(PB)^{3}$	5.9	7.1 (2)	6.2 (1)
	$B^{3}(PB)^{3}B$	6.2	7.2 (2)	6.9 (2)
	$B^{3}(PB)^{3}B^{2}$	6.4	7.0 (3)	7.0 (2)
JaOH-0566, $SMB^{3}MK^{62}$	0	5.3	8.3 (3)	7.9 (1)
	B	7.2	8.4 (3)	8.0 (1)
	B^{2}	7.0	8.5 (3)	7.5 (1)
	B^{3}	5.7	9.0 (3)	7.5 (2)
	B^{4}	7.0	8.9 (3)	8.8 (2)
	B^{5}	7.4	9.1 (2)	9.0 (2)
	$B^{5}PB$	4.7	8.9 (4)	7.9 (3)
	$B^{5}(PB)^{2}$	5.6	8.8 (3)	8.7 (2)
	$B^{5}(PB)^{3}$	5.7	9.4 (3)	8.7 (2)
	$B^{5}(PB)^{3}B$	7.8	9.2 (2)	9.0 (2)
	$B^{5}(PB)^{3}B^{2}$	8.5	8.6 (2)	8.8 (1)
	$B^{5}(PB)^{3}B^{3}$	8.8	9.0 (2)	8.6 (2)

[a] Titers are expressed as PFU/ml (log). Numbers in parentheses are days after infection when maximum titer of JEV was observed, examined for 5 days after infection.

[b] MB, adult mouse brain; SMB, suckling mouse brain.

[c] P, plaque isolation under agar overlay; B, growth of virus in BHK21 cells in fluid medium.

growth of the virus was observed between 22 and 37 C, although growth was much slower at 22 C, and a little faster at 37 C, than at 28 C (Fig. 2). At 40 C a slight increase of the virus PFU was followed by a rapid decrease, possibly due to virus inactivation and damage of host cells. Below 15 C, no significant increase of the virus PFU was observed.

The growth curve of virus at 28 C with an input multiplicity of 10 is shown in Fig. 3. The virus titer increased from 6 until 36 hr after infection. The virus titer in the culture fluid was always higher than that associated with cells.

DISCUSSION

Arboviruses are known to grow in arthropods to be transmitted by them to vertebrates. However, there have been relatively few studies on their in vitro cultivation in arthropod cells, although there have been many in mammalian and avian cells. In the case of mosquito-borne arboviruses, such as JEV, viruses were first cultivated in surviving tissues (Trager, 1938; Peleg and Trager, 1963) or in primary cultures (Peleg, 1968a) of insects. Detailed study on the growth of arboviruses including JEV in insect cells began after lines of insect cells had been established (Grace, 1962, 1966; Singh, 1967; Peleg, 1968b).

Suitor (1966) reported that JEV can multiply in Grace's moth cell line (Grace, 1962). Using this line or Grace's cell line from *Aedes aegypti* (Grace, 1966), Řeháček (1968a, b) showed limited growth and persistent infection with JEV. However, the maximum yields of JEV in these cell lines were less than 10^6 and 10^4 PFU/ml, respectively. Singh and Paul (1968a, b) showed that the cell line from *A. albopictus* (SA) of Singh (1967) was very susceptible to various kinds of arboviruses, including JEV, while the cell line from *A. aegypti* (SE) of Singh (1967) was much less susceptible. However, the maximum yield of JEV was less than 10^8 $TCID_{50}$ or LD_{50} per ml (Singh and Paul 1968a; Banerjee and Singh, 1968).

Our results show that of three lines of mosquito cells tested, SA gave the highest yield of JEV. The maximum yield of JEV in SA cells depended on the strain of JEV and its passage history. Using the JaOH-0566 strain, which had been passaged in MK and BHK, a reproducible, maximum yield of more than 10^9 PFU/ml was obtained in SA cells at 28 C. This seems to be the highest titer of JEV obtained so far in a tissue culture system. With this system, growth of JEV was observed in a temperature range of 22–37 C. Moreover, it is now possible to purify quantities of JEV starting from culture fluid of high titer from infected SA cells.

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