BIKEN JOURNAL Vol. 16, 57-66, 1973

DIFFERENCE IN PLAQUE SIZES OF JAPANESE ENCEPHALITIS VIRUS STRAINS ON BHK21 CELLS AND CHICK EMBRYO CELLS[1]

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(Received January 6, 1973)

SUMMARY The difference in the plaque sizes of Japanese Encephalitis Virus (JEV) strains on Baby Hamster Kidney (BHK21) cells and 10-day-old Chick Embryo (CE) cells was studied with the following results: 1) The difference in plaque size of JEV strains was found to be reproducible and the plaque size, as a biological characteristic, was stable at the 3rd-28th serial passage level in suckling mouse brain. 2) JEV strains were divided into three groups according to their plaque size on BHK21 cells, and designated as large type (3.9 to 5.1 mm diameter), intermediate type (3.1 to 3.8 mm) and small type (2.1 to 3.0 mm). 3) The difference in plaque size was not related to the date of isolation of the virus or the host sourse. 4) A correlation was found between the pathogenicity of JEV on subcutaneous inoculation into 23-25 day old mice and the plaque size on BHK21 cells. Large type strains were shown to be highly virulent to young adult mice. 5) The plaque forming abilities of JEV strains on CE and on BHK21 cells were compared but no correlation was found between the characteristics of their plaques on these two different species of host cells.

INTRODUCTION

It has been noted that JEV strains differ in biological properties, such as their optimal pH for hemagglutination (Okuno et al., 1965), interferon inducing ability (Rokutanda, 1969) and pathogenicity in young adult mice on extraneural inoculation (Nakamura and Nakamura, 1963: Oya et al., 1963).

Among animal viruses, variability of plaque size was first reported in polio virus strains by Dubes (1956), who divided the strains into small, intermediate and large plaque types. Variation in plaque size were also reported in wild strains and stock virus strains of Western equine encephalitis virus and these strains were also divided into three groups on the basis of plaque size (Marshall et al., 1962).

With regard to variability of plaque size of JEV, it has been reported that a strain adapted to mouse brain produced plaques on CE cells faster than when passaged in hamster kidney cells and that the former plaques were the larger (Inoue et al., 1961). It was found that

1 A summary of this work was reported at the 20th Annual Meeting of the Society of Japanese Virologists on November, 1972.

a strain attenuated by passage in hamster kidney cells produced smaller plaques than the wild strain (Rohitayodhin and Hammon, 1962: Nagai and Hammon, 1964).

Recently, Yamagishi and Yoshioka (1971) reported that the plaque forming abilities of the Nakayama-Yakken strain and JaGAr 01 strain on HeLa cells were different.

This paper describes studies on whether the difference in plaque size of JEV strains on BHK21 cells is a stable biological property. The plaque sizes of JEV strains and their plaque forming abilities on 10-day-old chick embryo cells and BHK21 cells, were also compared. Furthermore, the relationship between the plaque size of JEV strains on BHK21 cells and their pathogenicity to young adult mice was examined.

MATERIALS AND METHODS

1. *Virus strains*

Twenty-nine wild strains and 2 laboratory strains of JEV were used. They were isolated from mosquito, human, rabbit and swine, and some of them were kindly supplied by Dr. Oya, National Institute of Health of Japan.

The JaGAr 01 strain was used at the 9th passage in suckling mouse brain and other newly isolated strains at the 3rd-5th passage in suckling mouse brain, unless otherwise mentioned. The passage history of the Nakayama-NIH strain is not known in detail, but it was passaged at least 29 times in adult mouse brain and 6 times in suckling mouse brain. All viruses were prepared as 10% emulsions of infected suckling mouse brain in Tris-buffered saline, pH 7.4, containing 0.28% bovine plasma albumin (BPA). The emulsions were centrifuged at 10,000 rpm for 60 min in a refrigerated centrifuge and the supernatants were stored at −70 C as virus stocks.

2. *Tissue cultures*

Stable cell lines and primary culture cells were prepared for plaque assays using the procedures of Miles and Austin (1963) or Porterfield (1959). Growth medium for BHK21 cells consisted of one volume of Eagle's basal medium and one volume of YLE (Earle's BSS containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract) and 7% calf serum. An inoculum of 8 ml of cell suspension at a concentration of 5×10^5 cells/ml per 76 mm petri dish produced a monolayer in 48 hr at 37 C under an atmosphere of 5% CO_2 in air (CO_2 incubator).

Chick Embryo cells were prepared by trypsinization of 10-day-old eviscerated embryos. Growth medium for CE cells consisted of 0.5% lactalbumin hydrolysate, 0.002% phenol red in Earle's balanced salt solution (BSS) with antibiotics and 5% calf serum. Cell suspension (8 ml, 3.5×10^6 cells/ml) was placed in 76 mm petri dishes. The dishes were incubated at 37 C for 24 hr under an atmosphere of 5% CO_2 in air.

3. *Plaque assays*

The virus was diluted with the same buffer described above and a 0.4 ml aliquot of an appropriate dilution was inoculated onto a monolayer culture, which had been washed once with phosphate buffered saline. After an adsorption period of 90 min at 37 C in a CO_2 incubator, the monolayers were overlaid with 8 ml of first agar overlay medium, composed of YLE-medium and agar (Difco).

With CE cells, after incubation for 2 days at 37 C, 4 ml of second overlay medium (1 volume of 10-fold concentrated Earle's BSS, 1 volume of 1:1100 neutral red, 7.8 volumes of 1.25% agar and 0.2 volumes of 7.5% $NaHCO_3$) was added. Plaques were counted and their size was measured 40–42 hr after the second overlay.

For plaque assay on BHK21 cells, the first and second overlays were done with the same medium as for plaque assay on CE cells.

4. *Plaque count and measurement of plaque diameter*

Virus was diluted to give 30–60 plaques per dish. The size of plaques was measured with a slide caliper on at least 100 plaques of each strain. To avoid error due to delay in the time of measurement, the plaques were photographed, and plaque counts and plaque size were measured on the photographs. The diameter of plaques was measured in mm, approximating to the nearest 0.1 mm. The sizes of plaques at the edge of petri dishes and of fused plaques were not measured.

5. *Inoculation of young adult mice*

Young adult (23–25 day) mice were inoculated subcutaneously with 0.2 ml of virus suspension with a viral concentration of about $1.4\times10^5-6.8\times10^4$

PFU/ml. After inoculation, mice were observed daily for 20 days.

RESULTS

1. *Difference between three JEV strains in development of plaques on BHK21*

Fig. 1 shows the development of plaques of three JEV strains 3 to 9 days after inoculation onto BHK21. The JaOAr-363-70 strain produced relatively large plaques and the JaOAr-410-69 strain produced small plaques. The difference in plaque size increased with time after virus inoculation, being 0.7 mm on the 3rd day, 2.1 mm on the 5th day and 4.3 mm on the 7th day. Based on this result, in following experiments plaques were measured and counted on BHK21 cells 5 days after virus inoculation.

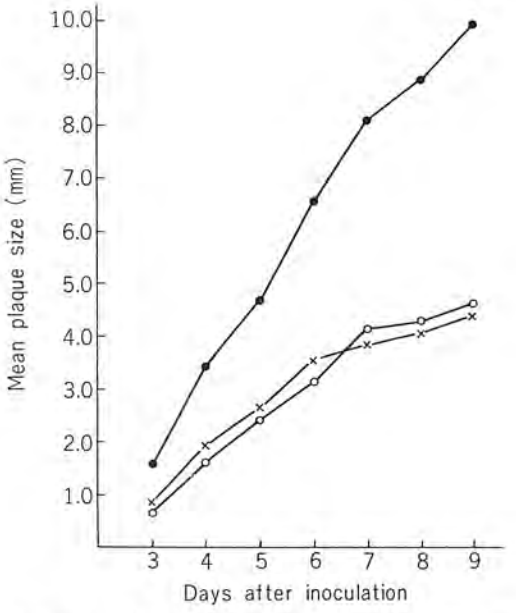

FIGURE 1. *Difference between JEV strains in development of plaques on BHK21 cells.* ●——●, *JaOAr-363-70;* ×——×, *JaOAr-410-69;* ○——○, *Nakayama-NIH.*

Fig. 2 shows photographs of large and small plaques formed on BHK21 by representative strains.

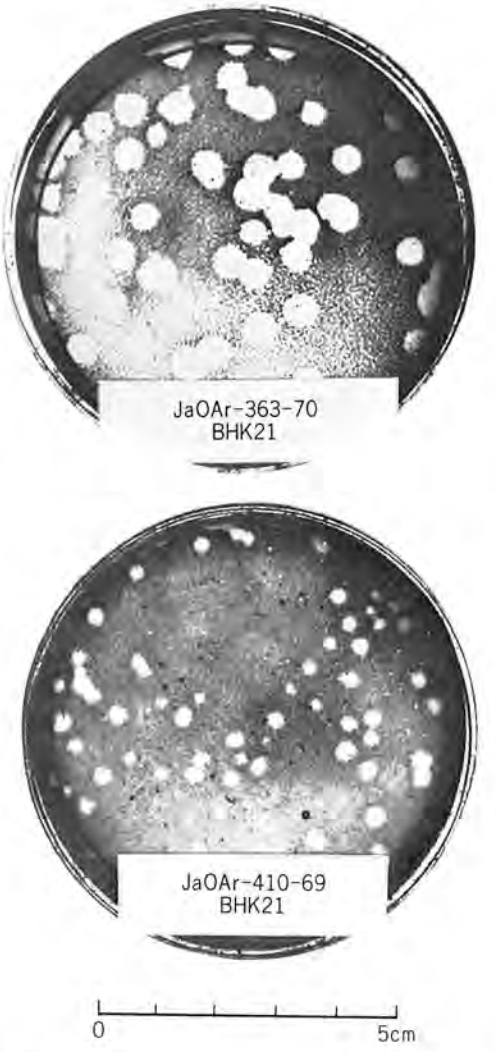

FIGURE 2. *Plaques produced by JEV strains on BHK21 cells 5 days after inoculation.*

2. *Effect of BHK21 cell density on plaque size and efficiency of infection*

Volumes of 8 ml of BHK21 cell suspensions at concentrations of 1.0×10^5 cells to 2.0×10^6 cells/ml were implanted into petri dishes. As shown in Table 1, the JaOAr-363-70 strain

TABLE 1. *Effect of BHK 21 cell concentration on plaque size and efficiency of infection*

Concentration of BHK 21 cells	Nakayama-NIH			JaOAr-410-69			JaOAr-363-70		
	Mean plaque size (mm)	Standard deviation	PFU/ml	Mean plaque size (mm)	Standard deviation	PFU/ml	Mean plaque size (mm)	Standard deviation	PFU/ml
1.0×10^5	2.0	0.4	7.5×10^7	2.4	0.4	9.5×10^7	3.1	0.7	1.4×10^8
2.5×10^5	2.4	0.4	1.3×10^8	2.8	0.5	1.5×10^8	4.2	0.6	2.2×10^8
5.0×10^5	2.2	0.4	1.4×10^8	2.4	0.4	1.8×10^8	4.2	0.5	2.5×10^8
1.0×10^6	2.2	0.4	1.5×10^8	2.3	0.4	1.6×10^8	4.2	0.6	2.5×10^8
2.0×10^6	2.0	0.4	2.1×10^8	2.2	0.4	1.3×10^8	3.9	0.7	2.8×10^8

which produced large plaques showed a slight decrease in plaque size at a concentration of 1.0×10^5 cells/ml, but the strains producing small plaques did not show any difference in plaque size at this cell concentration. No significant difference in plaque size or efficiency of infection was observed at initial cell concentrations of 2.5×10^5 to 2.0×10^6/ml. In the following experiments, BHK21 cell suspensions were adjusted to a concentration of 5×10^5 cells/ml.

3. *Reproducibility of plaque size, and relationship between passage level in suckling mouse brain and plaque size on BHK21 cells*

The constancy of the plaque sizes of the three JEV strains on BHK21 was examined. No significant difference was found between the mean values in three experiments or in the scattering of values (Table 2). The constancy of plaque sizes of the three strains during passage in suckling mouse brain was also examined in parallel experiments. As shown in Table 2, the plaque sizes of the strains were stable, even at as high passage levels as the 26th or 28th serial passage, and the sizes were highly reproducible in three experiments.

4. *Frequency distribution of the mean plaque sizes of JEV strains on BHK21*

Based on the reproducibility of the difference in plaque size of JEV strains on BHK21, the frequency distribution of the plaque sizes of 31 strains of JEV on BHK21 was studied.

TABLE 2. *Reproducibility of plaque size of JEV on BHK 21 cells at two or three passage levels in suckling mouse brain*

Strain	Passage level	Exp. 1		Exp. 2		Exp. 3	
		Mean plaque size (mm)	Standard deviation	Mean plaque size (mm)	Standard deviation	Mean plaque size (mm)	Standard deviation
JaGAr 01	9	2.6	0.4	2.5	0.5	2.9	0.4
	28	2.6	0.4	2.4	0.5	2.6	0.4
JaOAr-410-69	5	2.6	0.4	2.8	0.4	2.5	0.4
	11	2.8	0.5	2.4	0.4	2.3	0.4
JaOAr-404-68	5	4.7	0.5	4.5	0.5	4.2	0.6
	11	4.3	0.6	4.2	0.6	4.3	0.5
	26	4.4	0.6	4.4	0.6	4.3	0.7

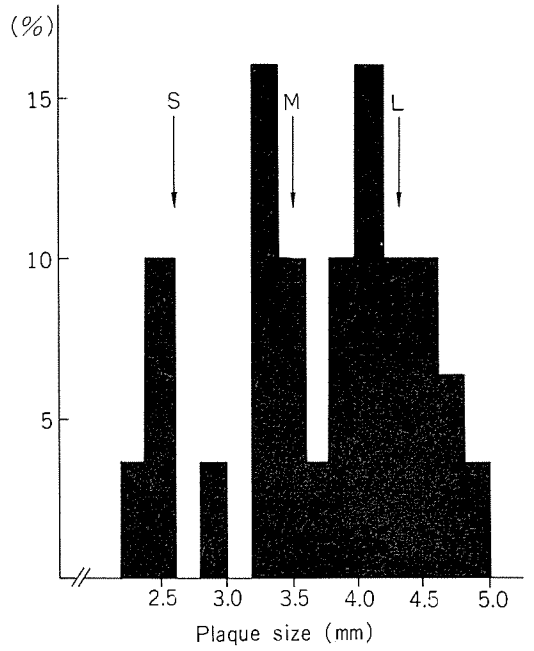

FIGURE 3. *Frequency distribution of mean plaque sizes of 31 strains of JEV on BHK21 cells.* ⟵, *mean diameter of plaques in each group.*

Fig. 3 shows that JEV strains can be divided into three groups on the basis of the mean diameter of their plaques. In the group producing large plaques (L type) plaques are 3.9 to 5.1 mm in diameter, in the intermediate group (M type) they are 3.1 to 3.8 mm and in the small group (S type) they are 2.1 to 3.0 mm in diameter.

5. *Comparison of plaque sizes of various strains*

As described above, JEV strains were clearly divided into three groups on the basis of the size of their plaques on BHK21. Next the relation of the plaque sizes of the 31 strains with the dates of their isolation and the hosts from which they were derived were examined. Swine and rabbits, as decoy animals, were also sources of the virus. Results are summarized in Fig. 4. There was no significant correlation of plaque size with the source or date of isolation of the strains.

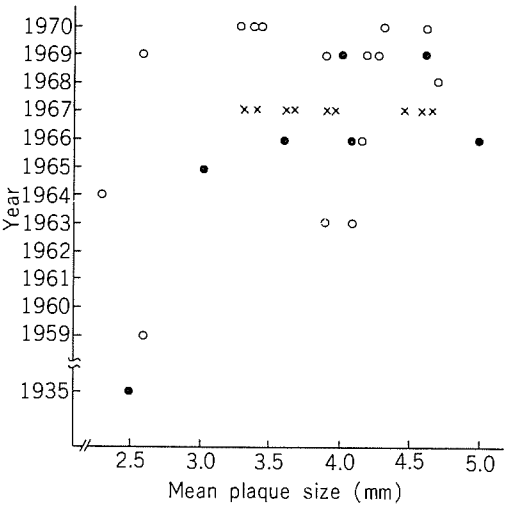

FIGURE 4. *Relation of plaque sizes of JEV strains with their sources and dates of isolation.* ○, *mosquito;* ●, *man;* ×, *pig and rabbit.*

6. *Correlation between plaque size on BHK21 and virulence of JEV strains to young adult mice*

The relation between the virulence of the strains to young adult mice and their plaque type on BHK21 was examined. Mice (ddY) of 23 to 25 days old were injected subcutaneously with virus and then observed for 20 days. The results are shown in Table 3. From the mortalities of the mice within 20 days after inoculation, the JEV strains were approximately divided into three groups, and this division was correlated with the plaque types on BHK21 except in the case of the JaGAr 01 strain. No mice died after inoculation of Nakayama-NIH or JaOAr-109-64 and only one of six mice died after inoculation with the JaOAr-410-69 strain, so these strains seemed to be weakly virulent to mice. These three strains were S type strains. Two of seven mice died when inoculated with the M type strains JaNAr-516-70 and JaGAr-17-70, so these strains seem to be moderately virulent. The mortalities with the L type strains were four of six mice with strain JaOAr-404-68 and four of five mice with strain JaOAr-363-70, so these strains appear to be highly virulent

TABLE 3. *Correlation between plaque size of JEV strains on BHK 21 cells and their virulence to young adult mice*[a]

Strain	Plaque type	Days after inoculation				
		5	8	11	13	20
Nakayama-NIH	S	0/6	0/6	0/6	0/6	0/6
JaGAr 01	S	0/7	2/7 (2)	5/7 (4)	7/7 (7)	
JaOAr-109-64	S	0/8	0/8	0/8	0/8	0/8
JaOAr-410-69	S	0/6	0/6	1/6 (1)	1/6 (1)	1/6 (1)
JaNAr-516-70	M	0/7	0/7	2/7 (1)	2/7 (2)	2/7 (2)
JaGAr-17-70	M	0/7	1/7	1/7 (1)	2/7 (1)	3/7 (2)
JaOAr-147-66	M	0/8	2/8 (1)	4/8 (2)	4/8 (3)	5/8 (5)
JaOAr-404-68	L	1/6 (1)	3/6 (2)	3/6 (3)	3/6 (3)	4/6 (4)
JaOAr-363-70	L	0/5	4/5 (2)	4/5 (4)	4/5 (4)	5/5 (4)
JaOH-05-66	L	0/8	3/8 (1)	4/8 (3)	5/8 (5)	5/8 (5)

[a] Denominator=number of mice inoculated. Numerator=number of mice showing typical symptoms plus number that died. Numbers in brackets indicate mice which died from viral infection.

to young adult mice. Moreover mice inoculated with L type strains tended to develop symptoms faster than those inoculated with S type strains.

7. *Distribution frequencies of plaque sizes of JEV strains on BHK21 and CE cells*

Fig. 5 shows the distribution frequencies of plaque sizes of JEV strains on two species of host cells, BHK21 and CE. The ratios of the mean plaque sizes on BHK21 to those on CE cells of the JEV strains were compared. The ratios for the Nakayama-NIH strain and JaGAr 01 strain were 0.71 and 0.85, respectively, both strains forming larger plaques on CE cells than on BHK21 cells. The ratio for the JaOAr–410–69 strain was approximately 1.0. The ratios for the JaOAr–363–70 and JaOAr–909–69 strains were highly being 2.15 and 2.05, respectively (Table 4). The data in Table 4 show no clear relation between plaque sizes on CE and on BHK21 cells.

Fig. 6 shows photographs of the plaques produced by the representative strains, Nakayama-NIH and JaOAr–363–70 on BHK21 and CE cells.

TABLE 4. *Comparison of plaque sizes and efficiencies of infection of Japanese encephalitis virus strains on chick embryo cells and BHK 21 cells*

Strain	CE cells			BHK 21 cells			(b)/(a)
	Mean plaque size (mm) (a)	Standard deviation	PFU/ml	Mean plaque size (mm) (b)	Standard deviation	PFU/ml	
Nakayama-NIH	3.5	0.6	1.1×10^{9}	2.5	0.4	1.4×10^{9}	0.71
JaGAr 01	3.4	0.5	8.8×10^{8}	2.9	0.4	8.7×10^{8}	0.85
JaOAr-410-69	2.6	0.5	5.3×10^{8}	2.6	0.4	6.0×10^{8}	1.00
JaGAr-17-70	2.3	0.4	6.3×10^{8}	3.3	0.5	8.0×10^{8}	1.43
JaOAr-63-63	2.7	0.3	2.3×10^{8}	4.1	0.6	2.8×10^{8}	1.52
JE-Mie-441-69	2.1	0.3	7.8×10^{8}	3.8	0.5	1.1×10^{9}	1.81
JaOH-05-66	2.7	0.4	5.3×10^{8}	5.0	0.5	4.5×10^{8}	1.85
JaNAr-516-70	1.7	0.3	8.0×10^{8}	3.4	0.6	1.2×10^{9}	2.00
JaYAr-458-70	2.3	0.5	5.0×10^{8}	4.6	0.5	6.5×10^{8}	2.00
JaOAr-909-69	2.1	0.4	5.0×10^{8}	4.3	0.5	9.5×10^{8}	2.05
JaOAr-363-70	2.0	0.3	8.3×10^{8}	4.3	0.6	1.3×10^{9}	2.15

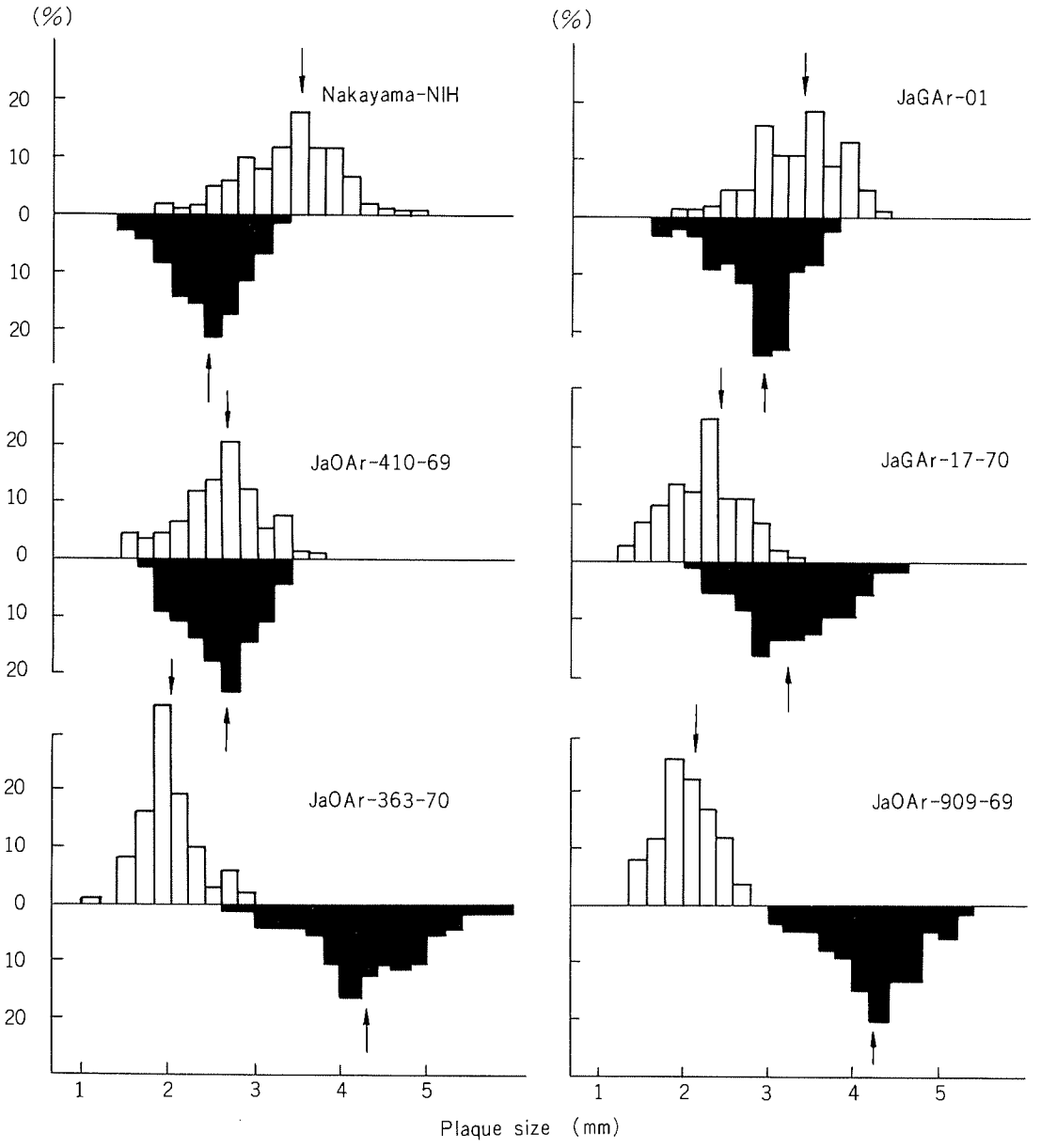

FIGURE 5. *Frequency distributions of plaque sizes of JEV strains on CE cells and BHK21 cells.* □, *chick embryo cells;* ■, *BHK21 cells;* ←—, *mean plaque size.*

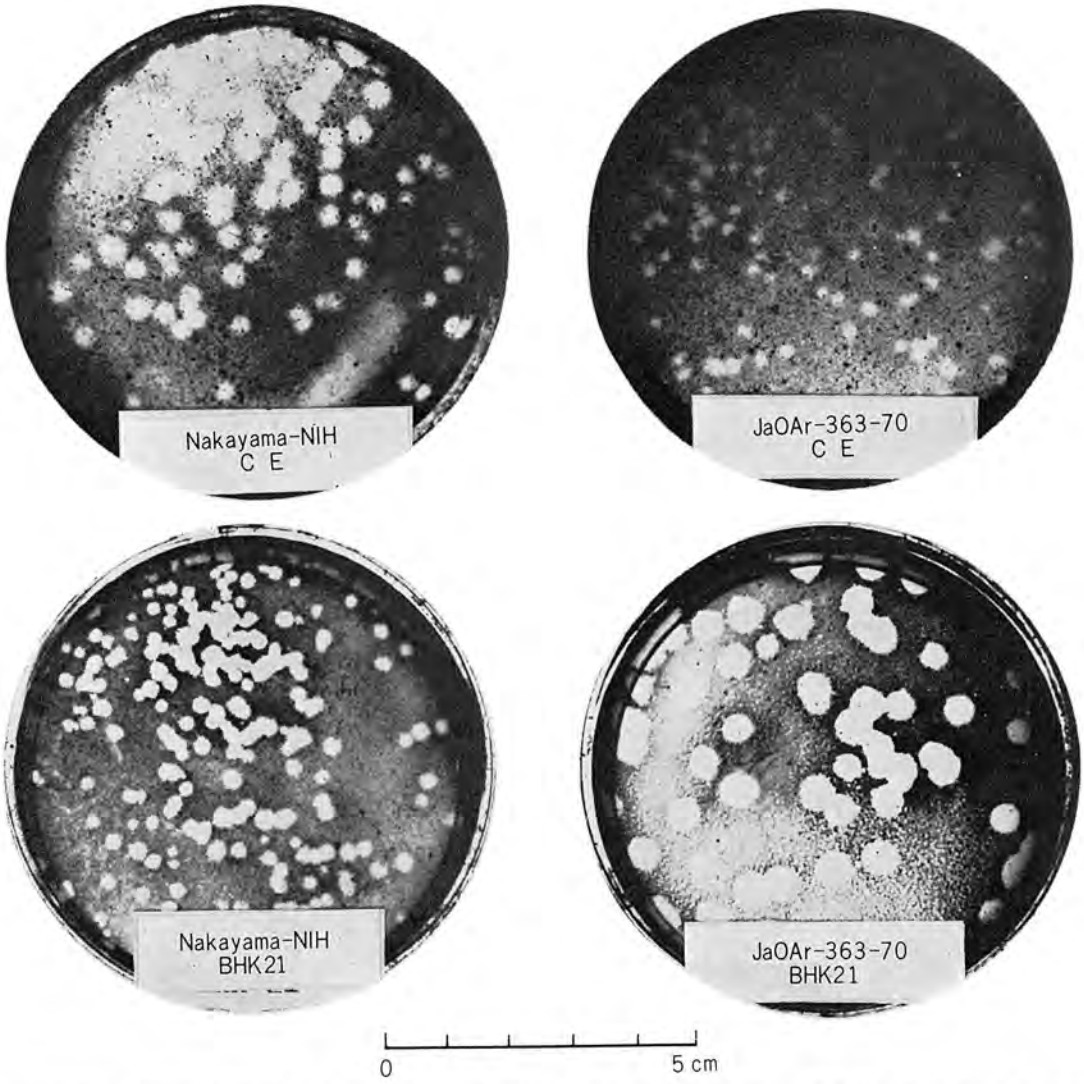

FIGURE 6. *Plaques produced by JEV, Nakayama-NIH and JaOAr-363-70 strains on CE cells and BHK21 cells.*

DISCUSSION

Previous reports on the morphology of plaques of animal viruses (Takemoto, 1969) show that different strains formed morphologically different plaques. Dubes (1956) grouped polio virus strains into three distinctly groups on the basis of plaque size. Moreover, Marshall reported that Western equine encephalitis virus could be classified into 3 groups from their plaque sizes on 10-day-old chick embryo cells.

In the present experiments, BHK21 cells were implanted at a concentration of 5×10^5 cells/ml, and differences in plaque size and efficiency of infection of JEV strains were investigated 5 days after virus inoculation. The plaque sizes of the strains were also ex-

amined after 26–28 passages in suckling mouse brain. Results showed that the plaque sizes were fairly stable and reproducible in duplicate tests (Tables 1 and 2). Thus the plaque size on BHK21 seems to be a characteristic, stable, biological property of strains of JEV.

Accordingly 31 strains of JEV were examined and were clearly divided into three groups on the basis of their mean plaque diameters (Large, Middle and Small types). No correlation was observed between the plaque size and the sources or dates of virus isolation. However, to confirm this a larger number of strains must be isolated and examined.

The correlation between plaque size and virulence of various animal viruses has been studied by many investigators. Zarate and Scherer (1969) reported that the plaque size of Venezuelan encephalitis virus in primary chick embryo cells was not correlated with its virulence to hamsters, mice or cotton rats. With all the JEV strains examined in this work, except JaGAr 01, the plaque size on BHK21 was found to be correlated with the virulence to mice, large type strains being more virulent (Table 3). These results suggest that the plaque size of JEV strains on BHK21 may be considered as a measure of the virulence of JEV to young adult mice. It has been observed with various animal viruses that reduction of plaque size is often associated with attenuation of the virus, and this has also been reported for JEV (Inoue and Kato, 1963; Nagai and Hammon, 1964). Some exceptions have also been found (Takemoto, 1966). We are now studying the correlation between plaque size of JEV strains on BHK21 cells and attenuation for young adult mice using the technique of repeated passage in vitro and in vivo.

The distribution frequencies of plaque sizes of JEV strains on two species of host cells, BHK21 cells and CE cells, were compared (Fig. 5). The ratio of the mean plaque size on BHK21 cells to that on CE cells was less than 1.0 with Nakayama-NIH and JaGAr 01, and more than 1.0 with JaOAr–363–70 and JaOAr–909–69. Similar results have been reported on the plaques of SV 40 virus strains on two kinds of host cells, AGMK cells and CV–1 cells (Takemoto, 1968). The reason for this phenomenon is unknown. The factors determining the plaque size of JEV strains on BHK21 are also unknown. It seems that the plaque size of virus on some host cells is influenced by the burst size and by one step growth. Other factors which seem to be related to the plaque size are the sensitivity of virus to interferon and culture conditions, such as the nature of the medium and temperature of incubation of infected cells. There is some evidence that a small plaque variant of Semliki forest fever virus was more susceptible to interferon and tended to produce more interferon than a large plaque variant (Finter, 1964), and that a small plaque variant of Sindbis virus was more susceptible to the action of interferon than a large plaque variant (Nagata et al., 1967). These results indicate that the concentration of interferon in infected cells may well influence plaque size. Studies on the factors influencing plaque size are now in progress.

ACKNOWLEDGEMENTS

We express our thanks to Drs. T. Kurimura and T. Kimoto for valuable advice.

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