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Plaque Studies with Certain Group B Arboviruses. I. Japanese B Encephalitis Virus Strains on Hamster Kidney and Chick Embryo Tissue Culture.* (29522)

KATSUJI NAGAI† AND W. McD. HAMMON
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa.

Extensive work with Japanese B encephalitis virus (JBE) has been carried out in several tissue culture systems, beginning about 1954 and later reviewed by Diercks and Hammon(1,2). The sensitivity of hamster kidney tissue cultures (HKTC) by CPE was shown to be equal or superior to that of mice. Nagai(3,4) demonstrated the propagation of this virus in several kinds of tissue culture, mostly primary tissue culture from chick embryo, pig, monkey and mouse origin; but no CPE was observed.

Plaque formation by JBE has been demonstrated in chick embryo tissue cultures (CETC)(5,6,7,8), hamster kidney(9,10) and duck kidney tissue cultures(11). Wallis *et al* (12) reported certain factors influencing enterovirus and reovirus plaque formation which, it seemed, deserved investigation in respect to JBE virus plaque growth. These included the method of sterilization of neutral red to avoid cell toxicity and the choice of agars to avoid viral inhibitors. Attempts have been made to evaluate these and other factors influencing plaque formation of JBE and to compare the plaque characteristics of 3 different strains of JBE virus under a variety of conditions on HKTC and CETC.

Materials and methods. a. *Virus strains.* The Nakayama strain used had been through 32 passages in HKTC following 47 mouse passages(1,2); the $TCID_{50}$ of the frozen pool used was $10^{-7.5}$/0.1 ml. The OCT-541-wild (OCT-wild) strain(13) was isolated directly in HKTC from wild caught mosquitoes and was used in this study in its third passage; the $TCID_{50}$ was $10^{-6.5}$/0.1 ml. The OCT-541 attenuated (OCT-attenuated) strain was derived by and had the properties described by Rohitayodhin and Hammon(14, 15,16); the TCD_{50} of the pool used was $10^{6.5}$/0.1 ml.

b. *Monolayer tissue cultures.* Primary tissue cultures of HKTC or CETC were prepared in 3 oz prescription bottles. Trypsinization was by a modification of the method of Bodian(17).

Outgrowth medium for HKTC consisted of 4% calf serum, 1% vitamin stock (Microbiological Associates, 100× concentration), 0.02 g% $NaHCO_3$ (0.5% of a 4% stock solution), 0.0292 g% L-glutamine (1% of a 2.92 g% stock), and 92.5% of a 0.5% solution of lactalbumin hydrolysate in Hanks' balanced salt solution (HBSS) and 1% antibiotic mixture. CETC medium consisted of 4% calf serum, 0.06 g% $NaHCO_3$, 93.5% lactalbumin hydrolysate in HBSS and 1% antibiotic mixture. Glutamine and vitamin stock were omitted.

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† Post Doctoral Fellow supported by Microbiology Training Grant 5 T 1 AI 110 from Nat. Inst. of Allergy and Infect. Dis. Member of faculty of Dept. of Pathology, Kyoto Prefectural Univ. of Med.

TABLE I. Comparison of Plaque Formation of Three JBE Virus Strains in HKTC Double Agar Overlay* at 37°C.

Virus strain	Plaque reading: Time in days†	No. of plaques‡	Diameter, cm	Plaque description
Nakayama	1/3	7.5	.3–.6	Clear, sharp boundaries round and ellipsoid
	1	7.5	.3–.7	*Idem*
	2, 3	8	.4–.75	"
OCT-wild	1/3	4	.3–.7	"
	1, 2, 3	4.5	.3–.7	"
OCT-attenuated	1/3	6.5	.1–.25	Hazy, sharp boundaries round and ellipsoid
	1	10.5	.1–.25	*Idem*
	2	14.5	.1–.25	"
	3	16.5	.1–.25	"

* 1.5% Bacto-agar in LAH with 0.003 g % autoclaved neutral red.
† Time after addition of second agar overlay on 8th day after inoculation.
‡ Avg of 2 bottles.

Maintenance medium for both types of cells was prepared as above, but contained only 2% calf serum, with $NaHCO_3$ increased to 0.1 g%. Routinely incorporated into all tissue culture media were 100 units of penicillin, 40 μg of streptomycin, 10 units of polymyxin and 25 units of nystatin per ml.

The first agar overlay was made by mixing equal parts (50 ml) of 3.0% agar (Difco Bacto-Agar) in sterile distilled water and the following: 2% lactalbumin hydrolysate in HBSS (4× concentrated, without either phenol red or $NaHCO_3$) 25 ml; calf serum 4 ml; $NaHCO_3$ (4%) 3 ml; antibiotics 1 ml; sterile water 17 ml. Difco Special Agar-Noble was employed in one series of comparative experiments. The second agar overlay medium was the same as the first except that neutral red was added. The neutral red was sterilized by autoclaving until comparative tests were made with filtered dye, after which the dye was routinely filtered through a Seitz pad. Single agar overlay when used with CETC was similar to this second overlay medium for HKTC.

c. *Plaque assay.* After a complete cell monolayer was obtained, maintenance medium was removed, the cultures were inoculated with 0.1 ml of appropriate dilution of virus, and incubated at 37°C with occasional rocking to improve distribution of the virus and prevent drying of the cells. For HKTC, after a 1- or 2-hour period, 6 ml of first agar overlay was added to each bottle and incubation was carried out at 37°, 30° or 24°C. On the 4th to the 10th days of incubation the second agar overlay containing neutral red was added. The bottles were examined for plaques 8 hours, 1, 2 and 3 days after the second agar overlay was applied.

Bottles of CETC, after an adsorption period for the virus of 1 to 2 hours were overlayed with 8 ml of agar. Incubation was at 37°, 30° and 24°C. Inspection for plaques was made after 4, 6, 8 and 10 days.

In most experiments several dilutions of virus were used for each strain tested, but in the Tables only results of that dilution giving the optimal number of plaques for the purpose of the particular experiment are presented.

Results and discussion. a. *Comparison of plaque formation of three different strains of JBE in HKTC.* Since neutral red is highly toxic for HKTC and cannot be employed satisfactorily in a single agar overlay system, double agar overlay technique was performed to compare plaque formation by Nakayama, OCT-wild and OCT-attenuated strains. The neutral red was autoclaved. Preliminary experiments showed that 8 days between the first and the second overlay was optimum for plaque formation at 37°C.

Table I shows the plaque counts 8, 24, 48 and 72 hours after the second overlay. Nakayama and OCT-wild strains produced round, clear plaques with sharp boundaries of 0.3-0.75 cm in diameter. The final reading could be made from 24 to 48 hours after the second agar overlay for these 2 strains.

TABLE II. Effect of Adsorption Time and Incubation Temperature on Number of Plaques Produced by 2 Strains of JBE Virus in HKTC.*

Virus strain	Temp of incubation, °C	No. of plaques† after different adsorption times at 37°C 30 min	60 min	120 min	150 min
OCT-wild‡	37	11	14	15	12
	30	4	5	9	8
	24	2	2	5	4
OCT-attenuated§	37	25	31	29	23
	30	22	32	28	25
	24	7	10	8	7

* 1.5% Bacto-agar in LAH with 0.003 g % autoclaved neutral red.
† Number using 1 bottle per time and temperature.
‡ Second agar overlay added after 6 days.
§ Second agar overlay added after 8 days.

The plaques produced by OCT-attenuated were small and hazy (0.1-0.25 cm) in contrast to those of the other 2 strains. The size did not increase after the 8-hour reading, but the number of plaques kept increasing through the later intervals of reading.

b. *Virus adsorption period and different temperature of incubation in HKTC.* OCT-wild and OCT-attenuated strains were compared for optimal adsorption time (30, 60, 120 and 150 minutes) at 3 different temperatures (37°, 30° and 24°C). The monolayers were not washed before adding agar. Results in Table II indicate that for OCT-wild 2 hours is about the optimal adsorption time at 37°, 30° and 24°C, though 1 hour may be adequate at 37°C, while 1 hour is apparently sufficient at each temperature for the OCT-attenuated strain. For the wild strain more plaques were formed at 37°C than at lower temperatures, but the attenuated strain formed as many or more at 30° as at 37°C.

Rhim and Hammon(9) with the Nakayama strain showed most effective adsorption at 35°C when comparing 30°, 35° and 40°C, and 2 hours gave the most complete adsorption. Miura and Scherer working at 36° with CETC and another JBE virus strain obtained maximal adsorption after 90 minutes (8).

c. *Virus adsorption period at 37°C in CETC.* Nakayama and OCT-attenuated strains were compared for the optimal adsorption time required at 37°C on CETC (single agar overlay). Table III shows that 90 minutes appeared to be optimal adsorption time for Nakayama strain while any time from 60 to 120 minutes produced nearly the same number of plaques for OCT-attenuated strain. Longer periods of adsorption led to fewer plaques, for reasons not readily understood. Results in Table II with HKTC suggested this same effect.

TABLE III. Effect of Adsorption Time on Number of Plaques Produced by 2 Strains of JBE Virus in CETC* at 37°C.

Virus strain	Adsorption time at 37°C, min	No. of plaques†
Nakayama	30	7
	60	11
	90	22
	120	12.5
	150	12.5
OCT-attenuated	30	21
	60	45
	90	47.5
	120	46
	150	37.5

* Single agar overlay; 1.5% Bacto-agar in LAH with 0.003 g % autoclaved neutral red.
† Avg of 2 bottles.

d. *Effect of neutral red concentration and different incubation temperatures on plaque formation with HKTC.* Different concentrations of neutral red (0.003 and 0.006%) were added in the second agar overlay in tests with all 3 strains of JBE in HKTC. Plaque numbers and size were not significantly affected by the concentration of neutral red, although with 0.003% the contrast was less than with higher concentrations.

Results with 0.004% neutral red are presented in Table IV. At 37°C incubation, all 3 strains of JBE produced larger plaques than at 30° and 24°C. The clearness of plaques was observed to be independent of the temperature of incubation for Nakayama and OCT-wild, but for OCT-attenuated, clear plaques were observed at 30° and 24°C while they were hazy at 37°C.

The plaque count was most readily made for Nakayama strain after 1-2 days at 37°C, 2-4 days at 30°C and 3-5 days at 24°C. In the case of OCT-wild, plaques could be counted most easily after 1-3 days at 37°C,

TABLE IV. Plaque Count and Size at Different Incubation Temperatures in HKTC* for the Nakayama, OCT-Wild and OCT-Attenuated Strains.

Virus strains	Incubation temp, °C	No. days after 2nd agar overlay	Plaque reading: No. of plaques†	Plaque reading: Diameter, cm	Plaque description
Nakayama	37	2	11.5	.3 –.6	Clear, sharp boundaries round and ellipsoid
	30	3	20	.1 –.2	*Idem*
	24	4	16	.1 –.2	"
OCT-wild	37	2	2	.3 –.5	"
	30	4	4	.1 –.3	"
	24	6	7	.15–.3	"
OCT-attenuated	37	4	6	.15–.4	Hazy, sharp boundaries round and ellipsoid
	30	5	9	.15–.25	Clear, sharp boundaries round and ellipsoid
	24	5	6	.1 –.2	*Idem*

* Second agar overlay added after 6 days; 1.5% Bacto-agar in LAH with 0.004% autoclaved neutral red.

† Avg number using 2 bottles per time and temperature.

4-5 days at 30°C and 5-7 days at 24°C. For the OCT-attenuated strain, the best plaque count was obtained after 3-5 days at 37°C and 4-6 days at 30° or 24°C.

e. *Effect of washing on plaque count of JBE virus in HKTC.* After the viruses were permitted 2 hours adsorption at 37°C on HKTC, the bottles were either not washed or were washed once, twice or 3 times with serum-free maintenance medium. For Nakayama and OCT-wild, as shown in Table V, washing more than once and possibly even once, appeared to affect plaque formation adversely and since the cells were observed to die early following repeated washings it is assumed that cell damage was responsible for reduced plaque formation. It would appear, therefore, that the first agar overlay should be added directly after the adsorption period, without any washing.

f. *Plaque formation in CETC at different temperatures.* Plaques were formed in CETC at 3 temperatures (37°, 30° and 24°C) using 2 hours adsorption at 37°C and *single* agar overlay (0.004% autoclaved neutral red). The Nakayama strain plaques at all temperatures were hazy, with sharp boundaries and never larger than 0.3 cm in diameter. Optimal times for reading were 5, 6 and 9 days at 37°, 30° and 24°C respectively. For the OCT-attenuated strain, plaques were hazy with sharp boundaries at 37° and 30° but clear at 24°C. Plaques could be read at the 8th day at any of these 3 temperatures. These plaques were similar in size to those of Nakayama strain.

With *double* agar overlay on CETC at these same temperatures plaque size for both Nakayama and OCT-attenuated was never larger than 0.2 cm. For the Nakayama strain the optimal temperature of incubation was

TABLE V. The Effect of Washing on Plaque Titers Following Adsorption of JBE, Nakayama and OCT-Wild Strains in HKTC* at 37°C.

Virus strain	Washing	No. days after 2nd agar overlay	No. of plaques†
Nakayama	None	2	11
		3	13.5
	1×	2	12
		3	14.5
	2×	2	13
		3	cells dead
	3×	2	" "
		3	" "
OCT-wild	None	2	7
		3	8
	1×	2	5
		3	6.5
	2×	2	5
		3	cells dead
	3×	2	" "
		3	" "

* Second agar overlay added after 6 days; 1.5% Bacto-agar in LAH with 0.003 g % autoclaved neutral red.

† Avg of 2 bottles.

TABLE VI. Effect of Bacto- and Noble-Agar* and Autoclaved and Filtered Neutral Red† on Plaquing Efficiency of JBE, Nakayama Strain in HKTC‡ at 37°C.

Overlay-agar and neutral red	No. days after 2nd agar overlay	No. of plaques§	Diameter of plaques in cm
Bacto-agar and autoclaved neutral red	4	18	.45–.7
	5	cells dead	
Bacto-agar and filtered neutral red	4	25.5	.4 –.7
	5	28.5	.4 –.7
Agar-Noble and autoclaved neutral red	2	20	.3 –.7
	3	cells dead	
Agar-Noble and filtered neutral red	3	21	.4 –.7
	4	cells dead	

* 1.5% Bacto-agar or special agar Noble in LAH.
† 0.003 g % neutral red.
‡ By double agar overlay technique; 2nd agar overlay added after 6 days.
§ Avg of 2 bottles.

37°C and the optimal day for the second agar overlay at this temperature was the 5th. For OCT-attenuated again, the highest plaque count was obtained at 37°C, but the second agar overlay gave best results on the 6th to the 10th days. Plaques were slightly larger when incubation was carried out at 30°C.

g. *Effect of agar and neutral red on plaquing of JBE virus.* Table VI presents the results of comparisons of Bacto-agar and Noble-agar (1.5%) and of filtered and autoclaved neutral red (0.003%) on the plaquing of JBE virus (Nakayama strain) by double agar overlay (second agar overlay after 6 days) in HKTC at 37°C.

Cells survived longer under the Bacto-agar and plaque numbers were slightly higher than with Noble-agar. Filtered neutral red appeared to have a similar advantage over autoclaved dye. The optimal number of plaques and the largest plaques were obtained by using Bacto-agar and filtered neutral red. Wallis *et al*(12) pointed out this advantage of filtering neutral red and of Bacto-agar in work with enteroviruses and reo virus. Repeated tests in this laboratory have confirmed these advantages with JBE virus in HKTC double overlay work.

Table VII shows the result of a similar experiment with the 2 types of agar and with 2 methods of neutral red sterilization on plaque production by the Nakayama strain in CETC at 37°C, using a single agar overlay. Essentially identical results were obtained with the OCT-wild and the OCT-attenuated strains in parallel tests. These results further support the advantages of Bacto-Agar and filtered neutral red for optimal plaque production by JBE virus.

h. *Effect of different concentration of $NaHCO_3$ on plaquing.* Table VIII shows the effect of different concentrations of $NaHCO_3$ on the plaquing of JBE (Nakayama and OCT-wild strains) in HKTC. It appears that $NaHCO_3$ concentration should be in the range of 0.06 g % to 0.1 g % to obtain the optimal number of plaques with either strain. In CETC, the same results were obtained with the Nakayama strain as in HKTC, *i.e.*, the optimal range was 0.06 g % to 0.1 g % $NaHCO_3$. Possibly 0.1 g % concentration was the optimum for plaque production.

TABLE VII. Effect of Bacto- and Noble-Agar* and Autoclaved and Filtered Neutral Red† on Plaquing Efficiency of JBE, Nakayama Strain in CETC‡ at 37°C.

Overlay-agar and neutral red	Plaque reading: After days	Plaque reading: No. of plaques§	Plaque reading: Diameter, cm
Bacto-agar and autoclaved neutral red	5	116	.1–.22
	6	cells dead	
Bacto-agar and filtered neutral red	6	132.5	.1–.23
	7	134.5	.1–.23
Agar-Noble and autoclaved neutral red	5	63	.1–.22
	6	cells dead	
Agar-Noble and filtered neutral red	6	69	.1–.22
	7	cells dead	

This plaque was hazy, sharp boundaries, round and ellipsoid.
* 1.5% Bacto-agar or special agar-Noble in LAH.
† 0.003 g % autoclaved or filtered neutral red.
‡ Single agar overlay.
§ Avg of 2 bottles.

TABLE VIII. Effect of Different Concentrations of $NaHCO_3$ on Plaque Count of JBE, Nakayama and OCT-Wild Strains in HKTC* at 37°C.

Virus strain	Conc of $NaHCO_3$ (g %)	No. of plaques†	Diameter of plaques in cm
Nakayama	.02	6	.3– .6
	.04	11	.3– .6
	.06	21.5	.3– .6
	.08	22	.3– .6
	.1	22	.3– .7
	.12	15	.3– .7
OCT-wild	.02	6	.4– .9
	.04	6.5	.5– .9
	.06	13	.5– .9
	.08	13.5	.5–1.0
	.1	18	.5–1.0
	.12	9.5	.4–1.0

* By double agar overlay technique; 2nd agar overlay added after 6 days and 1.5% bacto-agar and 0.003 g % filtered neutral red in LAH.
† Avg of 2 bottles.

Summary. Nakayama and OCT-wild strains of JBE produced similar plaques on HKTC, double agar overlay, which are 0.3-0.7 cm in diameter, clear with sharp boundaries while plaques produced by OCT-attenuated are much smaller and hazy.

Virus adsorption at 37°C is optimum at 2 hours for OCT-wild and only 1 hour for OCT-attenuated in HKTC; in CETC 90 minutes is the optimal adsorption time for Nakayama strain and 1 to 2 hours produces about the same plaques for OCT-attenuated strain. Washing after the adsorption period is apparently unessential and repeated washing is deleterious to the tissue. For optimal plaque visualization in HKTC with the 3 JBE strains, 0.004% neutral red is most satisfactory in the second agar overlay. Larger plaques occur with all strains at 37°C than at lower temperatures. Plaques produced by Nakayama and OCT-attenuated on CETC were much smaller and less clear than those on HKTC. Plaque formation of JBE Nakayama, OCT-wild and OCT-attenuated strains in HKTC (double agar overlay) and CETC (single agar overlay) is inhibited more by Noble-agar than by Bacto-agar. Autoclaved neutral red was less satisfactory than filtered dye for obtaining optimal plaques. $NaHCO_3$ in the concentration of about 0.1 g % gave the best plaques on both HKTC and CETC.

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