Plaque Studies with Certain Group B Arboviruses. II. Japanese B Encephalitis Virus on Hamster Liver Tissue Cultures.* (29597)

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The propagation of Japanese B encephalitis (JBE) virus *in vitro* using many different primary and continuous cell cultures has been described. JBE virus has also been shown to produce plaques under an agar overlay medium in hamster kidney tissue culture (HKTC), chick embryo tissue culture (CETC) and duck kidney tissue culture. Literature covering this previous work was recently reviewed(1). The present report describes the destruction, or cytopathogenic effect (CPE) of hamster liver tissue culture (HLTC) associated with propagation of the virus, and plaque formation in these cells.

Materials and methods. Virus strains. Four strains of JBE virus were used. The Nakayama strain had been through 33 passages in HKTC following 47 mouse passages. The frozen pool used in these experiments had a titer in HKTC of $10^{7.5}$ TCD_{50}/0.1 ml. The OCT-541-wild (OCT-wild) strain(2) was isolated directly in HKTC from field captured mosquitoes. The fourth passage in HKTC was used in these studies and had a titer of $10^{6.5}$ TCD_{50}/0.1 ml. The OCT-541-attenuated (OCT-attenuated) strain was that derived after repeated passage at 24°C as described by Rohitayodhin and Hammon (2,3). The TCD_{50} of the pool used was $10^{6.5}$ TCD_{50}/0.1 ml. The Peking strain isolated by Huang and Wang(4) was obtained in 1957 through the courtesy of Dr. Albert Sabin. Its titer was $10^{7.7}$ TCD_{50} in HKTC after one passage in this tissue.

Monolayer tissue culture. Monolayers of primary tissue culture, HKTC and HLTC, or a mixture of equal amounts of each, were made with slight variations of the method described by Bodian(5). Cultures were grown in 3 oz prescription bottles at 37°C. Secondary tissue cultures of HLTC were prepared by treatment with trypsin of cell monolayers grown in 16 or 32 oz prescription bottles. The medium was first removed from the cell monolayer, then the monolayer washed with 2.0 ml of prewarmed (37°C) 0.25% trypsin solution containing 1% of a 4.0% $NaHCO_3$ stock. After this washing an additional 5-10 ml of the trypsin solution was added to the monolayer, and incubated at 36°C for 3-5 minutes. The cells were removed from the glass by shaking the bottle and the total content aspirated with a pipette. This was centrifuged at 800 rpm for 10 minutes and the cells resuspended in outgrowth medium (0.1 ml packed cells/100 ml medium).

Outgrowth medium for HKTC was similar to that previously described(1) containing principally lactalbumin hydrolysate (LAH) in Hanks' balanced salt solution with 4% calf serum, with added vitamin stock, $NaHCO_3$, and L-glutamine. Medium for HLTC and mixed cultures was prepared as above, but contained 10% calf serum.

Maintenance medium for HKTC was the same as outgrowth medium but contained 2% calf serum and 0.1 g% $NaHCO_3$. For HLTC and mixed cultures the maintenance medium contained 4% calf serum and 0.1 g% $NaHCO_3$.

Outgrowth and maintenance medium for secondary tissue cultures were the same as for primary cultures.

Routinely incorporated into all tissue cul-

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ture 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 prepared without neutral red and the second overlay was prepared in a similar manner but 0.003% neutral red (Seitz filtered) was incorporated into the medium. The agar overlay contained 1.5% agar (Difco Bacto-Agar) in lactalbumin hydrolysate in HBSS (without phenol red), calf serum, antibiotic, and $NaHCO_3$ as previously described(1).

Plaque assay. After a complete cell monolayer was obtained, maintenance medium was removed and the cells inoculated with 0.1 ml of appropriate dilutions of virus. The bottles were then incubated at 37°C for 2 hours for adsorption with occasional rocking to improve distribution of the virus and prevent drying of the cells. To each bottle of cells inoculated with virus, 8 ml of the first agar overlay were added, and the bottles inverted after the agar solidified. The bottles were then incubated at 37°C or 30°C, and on the 6th or 10th day of incubation 8 ml of the second agar overlay containing neutral red were added.

Results and discussion. Cytopathogenic effect in HLTC. Normal HLTC and the sequential appearance of CPE in HLTC following inoculation with JBE virus strains is illustrated in Fig. 1 through 3.

CPE can be noted as early as 1-2 days after inoculation with virus dosages greater than 1000 TCD_{50}. Smaller inocula may not produce CPE before 4 or 5 days. When CPE occurs it proceeds to complete destruction. At first, a granular CPE is observed, which leads progressively to rounded, swollen cells which later slough from the glass. In contrast, HKTC infected with JBE virus demonstrate a granular type of CPE with small rounded cells, clustered in either small or large groups.

Comparative tube titrations of JBE virus strains on HLTC, HKTC and mixed cultures. Titrations were performed to compare the susceptibility of HLTC, HKTC and mixed culture to virus. The TCD_{50} titers were calculated by the method of Reed and Muench (6).

TABLE I. Results of Comparative Titration of JBE Virus on HLTC, HKTC and Mixed Culture.

Virus strains	Results of comparative titrations* HLTC† Primary	HLTC† Secondary	HKTC‡	Mixed§
Nakayama‖	7.5	7.0	7.5	7.5
OCT-attenuated¶	5.5	5.0	6.5	4.5
Peking**	7.5	6.5	7.7	—
OCT-wild***1	6.5	5.5	6.5	6.5
2	6.5	—	6.5	—
3	7.5	—	7.5	—

* All titers are shown as log TCD_{50}. Titration based on 2 tubes inoculated/dilution.
† HLTC = hamster liver tissue culture.
‡ HKTC = hamster kidney tissue culture.
§ Hamster liver and hamster kidney mixed culture.
‖ Nakayama mouse brain suspension passed 34 times in HKTC.
¶ OCT-attenuated strain grown at 24°C.
** Peking strain mouse brain suspension passed 1 time in HKTC.
*** OCT-wild strain 1 = 3 passages in HKTC; 2 = 3 passages in HKTC and 5 passages in HLTC; 3 = 3 passages in HKTC and 10 passages in HLTC.

Results are shown in Table I. By CPE titers, primary HLTC and mixed cultures were as sensitive as primary HKTC to the Nakayama, Peking and OCT-wild strains. However, the OCT-attenuated strain was slightly more pathogenic for HKTC, in which it had been previously passed many times, than for any of the other cultures. Secondary HLTC was less sensitive than primary HLTC for all viruses.

The titers of OCT-wild through 10 passages in HLTC were found to be similar in primary HLTC and in HKTC.

Comparison of plaque formation by 2 JBE virus strains in HLTC using double agar overlay. The double agar overlay technique was used with confluent monolayers of HLTC to demonstrate and compare plaque formation by Nakayama and OCT-wild strains. The second agar overlay with neutral red incorporated was added on the 6th day, and incubated at 37°C. Plaques were observed 6 days following the second overlay. Table II shows that similar plaques were obtained with the Nakayama strain and with the OCT-wild strain (passage 3 in HKTC and follow-

ing an additional 10 passages in HLTC). The plaques produced by both were clear with sharp boundaries and were 0.3-0.7 cm in diameter. Sensitivity for virus detection appeared to be somewhat lower than the CPE method for both virus strains. Ten passages of the OCT-wild strain in HLTC did not result in a significant increase in plaque titer though it had titered higher by the CPE method. One test at 37°C with the Nakayama strain in secondary HLTC (not included in table) gave plaque sizes slightly smaller than those shown for the primary tissue, and the plaques were hazy. However, the titer of the virus was slightly higher than that obtained by using primary cells. Negative results were obtained when both the single and double agar overlay techniques were used with confluent monolayers of mixed cell culture.

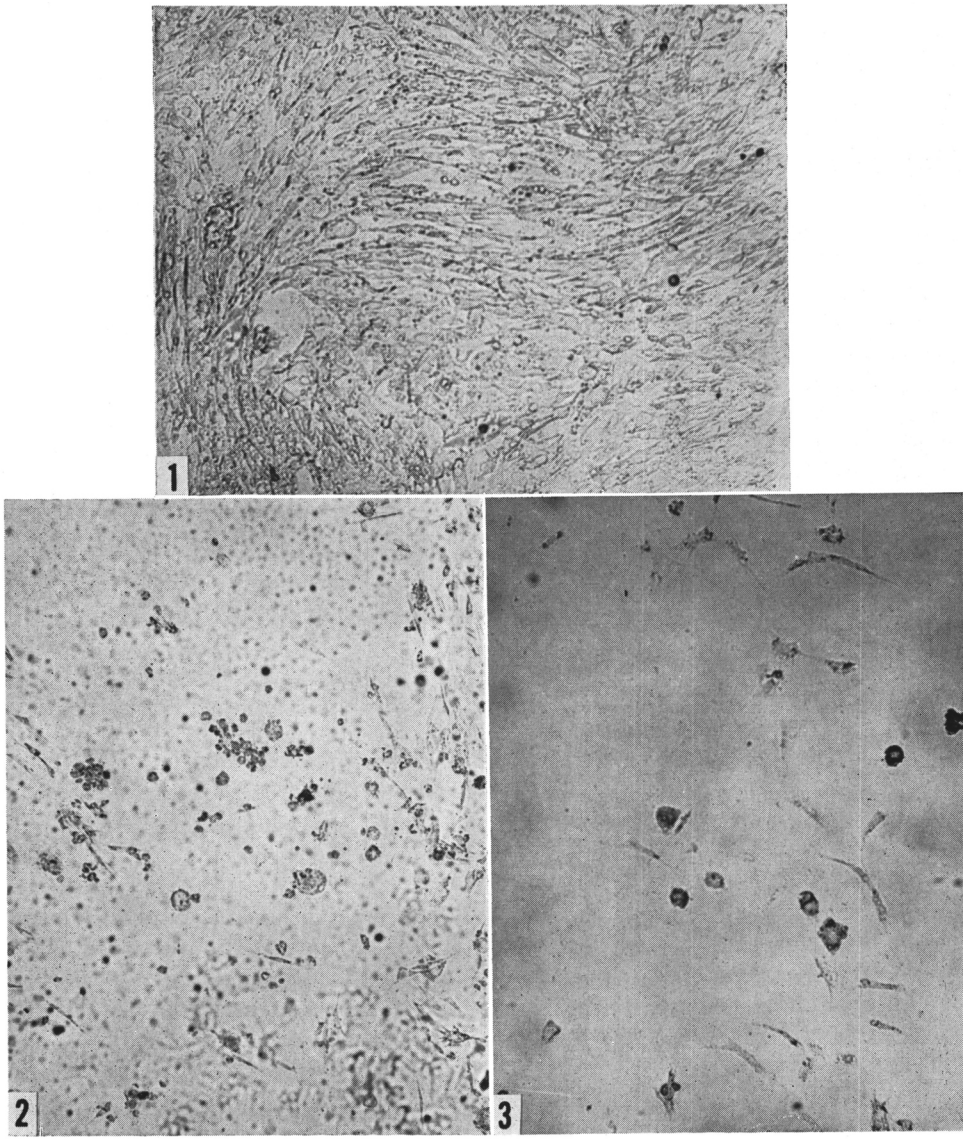

FIG. 1. Uninoculated normal hamster liver cells.
FIG. 2. Hamster liver cells infected with JBE virus, 3 days after inoculation of a 10^{-4} dilution.
FIG. 3. Hamster liver cells infected with JBE virus, 3 days after inoculation of a 10^{-2} dilution.

TABLE II. Comparison of Plaque Formation of 2 JBE Virus Strains in Primary HLTC by Double Agar Overlay.

Virus strains	Temp of incubation, °C	2nd Agar overlay system, day applied	Titer/0.1 ml PFU	Size, cm	Description
Nakayama*	37	6	18.5×10^5	.4–.7	Clear, sharp boundaries, round and ellipsoid
OCT-wild† 1	37	6	11×10^5	.4–.7	*Idem*
2	37	6	17.5×10^6	.3–.6	

* Nakayama mouse brain virus strain passed 34 times in HKTC.
† Oct-wild strains 1 = 3 passages in HKTC; 2 = 3 passages in HKTC, and 10 passages in HLTC.

Summary. 1. Monolayer hamster liver tissue cultures proved to be highly susceptible to JBE virus on the basis of CPE titers. Primary HKTC, HLTC and mixed cultures were found to be equally sensitive for several virus strains. Secondary HLTC was less sensitive than the primary. 2. Nakayama and OCT-wild strains of JBE produced similar plaques on HLTC using the double agar overlay technique at 37°C, but the method was not as sensitive as CPE for virus detection. Plaques were 0.4-0.7 cm in diameter and clear with sharp boundaries. 3. Plaques were produced on secondary HLTC using the double agar overlay technique at 37°C. These were smaller, and hazy but in increased numbers. Mixed kidney and liver cultures failed to produce plaques.

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Streptococcal Infection in Normal and Splenectomized Monkeys.* (29598)

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The question of post-splenectomy hypersusceptibility to infection has been undergoing a period of critical appraisal in various clinics. There is not general agreement as demonstrated by recent reports(1,2). Most experimental studies have been conducted in non-primates with varying results dependent on animal species and agent employed(3); relatively little work has been done with primates. Increased susceptibility of splenectomized monkeys to poliomyelitis(4) and plasmodium infection(5,6) have been suggested. Studies in this laboratory have been concerned with the comparative response of normal and splenectomized monkeys to a variety of agents. In this report are described the results following aerosol and intravenous challenge with streptococci.

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Materials and methods. Young pre-puber-