THE VIRAL RANGE IN VITRO OF A MALIGNANT HUMAN EPITHELIAL CELL (STRAIN HELA, GEY)

II. Studies with Encephalitis Viruses of the Eastern, Western, West Nile, St. Louis, and Japanese B Types *

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The successful propagation and cytopathogenic effects of the viruses of poliomyelitis, herpes simplex, pseudorabies, and vaccinia in cultures of human malignant epithelial cells cultivated *in vitro* (strain HeLa, Gey) have been reported.[1-4] The purpose of this article is to present information that relates to the destructive effects and multiplication in cultures of strain HeLa cells, of the arthropodal-borne encephalitis viruses, of Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis (WEE), West Nile, St. Louis encephalitis (SLE), and Japanese B encephalitis (JBE).

Materials and Methods

Viruses. The strain of EEE virus employed for these studies was isolated from a patient in 1951 by Dr. H. E. Dascomb, Louisiana State University. Infected mouse brain therefrom was passed intracerebrally once in mice in our laboratory for the preparation of a 5 per cent brain suspension in sterile 5 per cent dextrose in water. This brain suspension had an LD_{50} of $10^{-7.5}$/0.03 to 0.05 ml.

WEE virus and the Hubbard strain of SLE virus were obtained from the State of Minnesota Public Health Laboratory as 10 per cent suspensions of infected mouse brain. A single passage of WEE virus intracerebrally in mice yielded a 10 per cent suspension of brain tissue.

Lyophilized West Nile virus, labeled "25th passage, 10% MB, J 725960 in NMS, April 27, 1942 IHD lab.," available by courtesy of Dr. K. C. Smithburn, was purchased from the American Type Culture Collection. One passage intracerebrally in mice was carried out to make a 10 per cent suspension of infected brain.

A sample of JBE virus, Nakayama strain, as frozen and dried mouse brain, "5–17–48," was supplied by courtesy of Dr. C. M. Eklund. The virus employed was contained in 10 per cent suspension from the second mouse passage in our laboratory.

Viruses were stored in sealed ampoules at −70° C.

The methods for *viral assay, cellular cultivation, viral propagation,* and *photography* were described in the preceding article of this series.[4]

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Part I of these Studies is the immediately preceding article. Part III will appear in the January–February issue and will include a general discussion of the group. Part I should be consulted for explanation of certain abbreviations.—*Editor.*

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Experimental Results

The purposes of these experiments were to learn (a) whether the virus under study would affect the morphologic characteristics of strain HeLa cells and (b) whether the virus would propagate. To accomplish these objectives, serial passage of virus was carried out in test tube or Porter flask cultures and microscopic observations of cells were made daily. The presence of virus in a culture was established by intracerebral inoculation of cultural liquid into mice and/or by the occurrence of cytologic changes in strain HeLa cells.

Eastern Equine Encephalomyelitis Virus

Cytologic Effects of EEE Virus. The destructive effects of EEE virus for strain HeLa cells became evident during the first passage of virus. To permit recording of these cytologic changes photographically, one passage was performed in Porter flask cultures.

Experiment 1. Two Porter flask cultures were prepared for viral inoculation by washing the cells and glass surfaces twice with MS–100, 1.0 ml. Chicken serum, 10 per cent, and maintenance solution, 90 per cent (CHS–10, MS–90), 1.0 ml., and EEE virus, 0.1 ml., from the 12th passage of experiment 2, were added to each flask. The fluid was removed from each flask before cells were photographed. It was replaced after photography when further incubation was desired. Photographs of cells from this experiment are shown in Figures 1 to 4.

The appearance of strain HeLa cells from a culture infected by EEE virus (Figs. 2 and 4) is in striking contrast to the appearance of cells in uninfected cultures (Figs. 1 and 3). This virus rapidly destroyed strain HeLa cells with the result, within 48 hours, of the disappearance of most cells from the glass (Fig. 2). However, a few cells often were spared and retained their normal shape for several additional days (Fig. 4).

Multiplication of EEE Virus. Serial passage of EEE virus in cultures of strain HeLa (experiment 2) was attempted to learn whether the virus would propagate.

Experiment 2. EEE virus, 0.05 ml., as mouse brain suspension, was inoculated into each of two test tube cultures of strain HeLa cells, containing 0.5 ml. of maintenance solution, 100 per cent (MS–100).* The cultures for this experiment were not rinsed with balanced salt solution since antibodies for EEE virus were assumed not to exist in the human serum employed for cellular cultivation. The

* Maintenance solution (MS–100) without chicken or other serum, 10 per cent, maintains strain HeLa cells well for at least 3 to 4 days at 37° C., if the old serum-containing medium used during the period of cellular cultivation is not removed completely by rinsing the cells and cultural vessel with salt solution. MS is enriched sufficiently to permit cellular maintenance without non-specific degeneration by the small amount of serum medium that remains in the vessel when the supernatant liquid is merely replaced.

medium containing serum was merely removed and replaced with MS–100. When degeneration of cells occurred, the supernatant liquids from two tubes were pooled, and aliquots, 0.05 ml., were transferred to each of two uninfected cultures to effect serial passage of virus. The data from experiment 2 are given in Table I.

The results of experiment 2 (Table I) show that EEE virus multiplied in cultures of strain HeLa cells. Virus infectious for mice and destructive for strain HeLa cells persisted through 20 serial passages,

TABLE I

Propagation in Vitro of Eastern Equine Encephalomyelitis Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Results as indicated by		
			Cytopathogenic effect	Infectivity log of cultural liquid for	
				Mice*	HeLa cells†
Inoculum				7.5	
1	2	1.0	2/2‡		
5	12	5.0	3/3		
10	22	10.0	3/3	5.0	4.0–5.0
15	35	15.0	2/2		
20	48	20.0	3/3	>3.0	5.0–6.0

* The results of mouse titrations in all tables are expressed as the negative log of the LD_{50}/0.05 ml. of diluted cultural liquid.

† The results of strain HeLa titrations are given as the negative log of the dilution of cultural liquid which, per 0.4 ml., produced a specific viral cytopathogenic effect, in a tube culture of cells, after 4 to 7 days of incubation at 36° C.

‡ For each of the tables, the numerator signifies the number of cultures that showed a viral cytopathogenic effect or the number of mice that died from viral infection. The denominator indicates the number of cultures or mice inoculated with virus.

over 48 days. The original virus inoculum (LD_{50}, $10^{-7.5}$) was thus diluted 10^{20} times. The results of titrations in strain HeLa cultures and in mice at the 10th and 20th passages add evidence that virus multiplied.

Western Equine Encephalomyelitis Virus

Cytologic Effects of WEE Virus. A cytopathogenic effect for WEE virus was uniformly obtained by the use of mouse brain suspensions although virus from several subsequent passages (experiment 4) failed to destroy strain HeLa cells. To learn whether this cellular destruction was specifically related to virus, neutralization with specific antibody was attempted (experiment 3). For photographic purposes, Porter flasks were used.

Experiment 3. Porter flask cultures were prepared for viral inoculation by washing with balanced salt solution and by the addition of CHS–10, MS–90, 1.0 ml. as in experiment 1. WEE virus, 0.1 ml., as 2 per cent suspension of infected

mouse brain was added to each of four flasks. Human serum containing antibodies for WEE virus, kindly supplied by the Minnesota State Public Health Laboratories, was added to two cultures as 0.1 ml. of undiluted serum. Photographs that present the results of this experiment are shown in Figures 5 and 6.

A cytopathogenic effect of WEE virus for strain HeLa cells was seen 1 day after addition of virus (Fig. 5). In contrast to the nearly complete destruction of cells by WEE virus (Fig. 5), was the normal appearance of cells in the culture inoculated with specific antibody and virus (Fig. 6). The prevention of cellular destruction by the use of specific antibody established a causal relationship between WEE virus and its cytopathogenic effect for strain HeLa cells.

Multiplication of WEE Virus. The purpose of experiment 4 was to learn whether WEE virus would grow in cultures of strain HeLa cells.

Experiment 4. Serial passage of WEE was carried out in a manner similar to that for EEE virus (experiment 2). However, the cultures in this experiment were prepared for virus inoculation by washing the cells and walls of the cultural vessels twice with MS–100, or Hanks's salt solution, 0.5 ml., before the addition of CHS–10, MS–90, 0.5 ml. Virus was passed when destruction of cells occurred, or at approximately weekly intervals. The viral inoculum for the first passage was 0.05 ml. of 10 per cent suspension of infected mouse brains. The results of this experiment are given in Table II.

Data related to the propagation of WEE virus in cultures of strain HeLa are shown in Table II. Ten serial passages of virus, extending

TABLE II

Propagation in Vitro of Western Equine Encephalomyelitis Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Results as indicated by		
			Cytopathogenic effect	Infectivity for mice	Titration in mice
1	2	1.0	2/2		
2	5	2.0	2/2	5/5	4.7
4	23	4.0	0/2	5/5	4.5
9	45	9.0	2/2	5/5	
10	50	10.0	2/2	5/5	

over a 50-day period, resulted in a 10^{10} dilution of the original viral inoculum. Fluids from passages 2 to 6, 9, and 10 were tested in mice and produced neurologic disease and death. Destruction of strain HeLa cells was seen in passages 1, 2, 5, and 7 to 10. In passages 3, 4, and 6, no cellular destruction occurred even though the cultures were incubated for longer periods than were used for other passages (11, 7, and 8 days, respectively). A reason for these failures of the virus to cause cellular damage has as yet not been learned.

West Nile Virus

Cytologic Effects of West Nile Virus. The cytopathogenic effect of West Nile virus for strain HeLa cells was seen in the first passage of virus. These cellular changes were recorded photographically in Porter flask cultures (experiment 5).

Experiment 5. The procedure for this experiment was similar to that for experiment 3 with WEE virus. The inoculum was a 10 per cent suspension of infected brain tissue from the first mouse passage of lyophilized virus. The cellular changes are shown in Figures 7 and 8.

West Nile virus destroyed strain HeLa cells (Figs. 7 and 8) though this cytopathogenic effect was usually delayed for at least 5 or 6 days after virus inoculation.

Multiplication of West Nile Virus. The ability of strain HeLa cells to support propagation of West Nile virus was tested in two experiments. For one (experiment 6), reconstituted lyophilized virus purchased from the American Type Culture Collection was used to inoculate the first passage cultures; for the other (experiment 7), a 10 per cent suspension of infected mouse brains from the first mouse passage of lyophilized virus was employed.

Experiments 6 and 7. The procedures for experiments 6 and 7 were similar to that employed for experiment 4 with WEE virus. The approximate dilutions of human serum medium effected by washing the cultures were $10^{3.3 \text{ to } 3.6}$ for passages 1 to 3; $10^{3.3}$ for passage 4 of each experiment; $10^{4.6}$ and $10^{5.6}$ for passage 5 of experiments 6 and 7, respectively; $10^{5.2}$ and $10^{6.2}$ for passage 6, and $10^{4.9 \text{ to } 5.2}$ for passages 7 to 12. The results of experiments 6 and 7 are given in Table III.

Evidence for the multiplication of West Nile virus in strain HeLa cells is shown in Table III. Virus was carried through twelve serial

TABLE III

Propagation in Vitro of West Nile Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture		Cumulative log of dilution of original viral inoculum	Results as indicated by					
				Cytopathogenic effect		Infectivity for mice		Titration in mice	
	Exp. 6	Exp. 7		Exp. 6	Exp. 7	Exp. 6	Exp. 7	Exp. 6	Exp. 7
1	6	5	1.0	2/2	2/2				
3	24	18	3.0	1/2	2/2	5/5	5/5	2.4	3.9
5	36	29	5.0	0/2	0/2	5/5	5/5		3.5
10	72	64	10.0	2/2	2/2	6/6	6/6	1.6	1.6
12	100	95	12.0	2/2	2/2	5/5	5/5		

passages in experiments 6 and 7, over periods of 100 and 95 days. The virus was uniformly infectious for mice. Destructive effects of West

Nile virus for strain HeLa cells were observed in all passages except 4, 5, and 6 in each experiment. Since passages 4, 5, and 6 in both experiments were carried out simultaneously, a common factor may have caused the absence of cytopathogenic effects.

St. Louis Encephalitis Virus

Cytopathogenic effects of SLE virus for strain HeLa cells were observed infrequently and irregularly. Yet, the virus multiplied.

Multiplication of SLE Virus. Serial passage of SLE virus was attempted in cultures of strain HeLa to determine whether the virus would multiply.

Experiment 8. The procedure for this experiment was similar to that employed for experiment 4 with WEE virus. The cultures for the first seven passages of this virus were washed to result in approximately a $10^{3.3}$ dilution of the human serum used in the medium for cellular cultivation; for passages 8 and 9 an approximate dilution of $10^{5.2}$ was effected. The inoculum for the first passage cultures was 0.05 ml. of 10 per cent suspension of infected mouse brain. The data from this experiment are given in Table IV.

Table IV presents evidence to show that SLE virus propagates in cultures of strain HeLa cells. Nine serial passages of virus, extending over a 72-day period, were carried out successfully. The pooled cultural

TABLE IV

Propagation in Vitro of St. Louis Encephalitis Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Results as indicated by		
			Cytopathogenic effect	Infectivity for mice	Titration in mice
1	6	1.0	2/2		
2	25	2.0	0/2	5/5	2.4
5	44	5.0	0/2	5/5	3.6
9	72	9.0	0/2	6/6	

liquids from each passage produced in mice signs of encephalitis and death, 4 or 5 days after intracerebral inoculation. Confirmatory evidence for propagation of SLE virus was obtained from the results of titrations of fluid from the second and fifth passages.

SLE virus commonly did not exert a cytopathogenic effect for strain HeLa cells; destruction of cells was observed only in passages 1, 6, and 7. In other passage cultures, the cells retained a normal appearance for periods of from 6 to 19 days, despite the presence of virus as shown by mouse inoculation.

Japanese B Encephalitis Virus

Serial passage of JBE virus in cultures of strain HeLa cells was attempted to learn whether these cells were capable of propagating virus and/or of responding to infection by cytologic changes.

Experiment 9. Serial passage was initiated with JBE virus in 10 per cent mouse brain tissue, 0.1 ml., as the inoculum for each of two rinsed cultures of HeLa cells containing 0.9 ml. of CHS–10, MS–90. For each successive transfer at intervals not greater than 7 days, or when cytologic changes were observed, the supernatant fluids of the two cultures were removed, pooled, and an aliquot, 0.1 ml., was transferred to each of two tubes of HeLa cells. The data of this passage series are given in Table V.

TABLE V

Propagation in Vitro of Japanese B Encephalitis Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Results as indicated by		
			Cytopathogenic effect	Infectivity for mice	Titration in mice
Inoculum				5/5	
1	5	1.0	2/2	1/4	
2	9	2.0	14/14	Not tested	
4	21	4.0	2/2	Not tested	
5	27	5.0	0/2	0/4	
10	61	10.0	0/2	5/5	2.3
13	66	13.0	0/2	6/6	

The data in Table V show that virus persisted through 13 passages over a period of 66 days resulting in dilution for the initial virus inoculum of a cumulative log of 13 or more. Overt evidence for cytopathogenic effect was present for 4 passages in the total destruction of all cells in from 4 to 6 days. During passages 5 to 13, most of the cells retained their normal structure; the attrition that results from such factors as age, nutrition, and low pH was held responsible for the degenerative and dead cells in cultures.

SUMMARY

Human epithelial cells (strain HeLa, Gey), cultured *in vitro* since their derivation from an epidermoid carcinoma of the cervix in February, 1951, were found to support the multiplication of encephalitis viruses of the Eastern, Western, West Nile, St. Louis, and Japanese B types. Eastern, Western, and West Nile types of virus regularly produced specific destruction of strain HeLa cells. It was shown with Western equine encephalomyelitis virus that cultures of strain HeLa

cells can be utilized for the demonstration of specific antibodies for the cytopathogenic encephalitis viruses. Cultures of strain HeLa cells were employed for titration of Eastern equine encephalomyelitis virus.

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LEGENDS FOR FIGURES

All photographs show unstained cells.

FIG. 1. Strain HeLa cells kept at 36° C. for 1 day in CHS–10, MS–90, and photographed immediately before inoculation of virus. × 150.

FIG. 2. Strain HeLa cells, photographed 2 days after viral inoculation to show the destructive effects of Eastern equine encephalomyelitis virus. × 150.

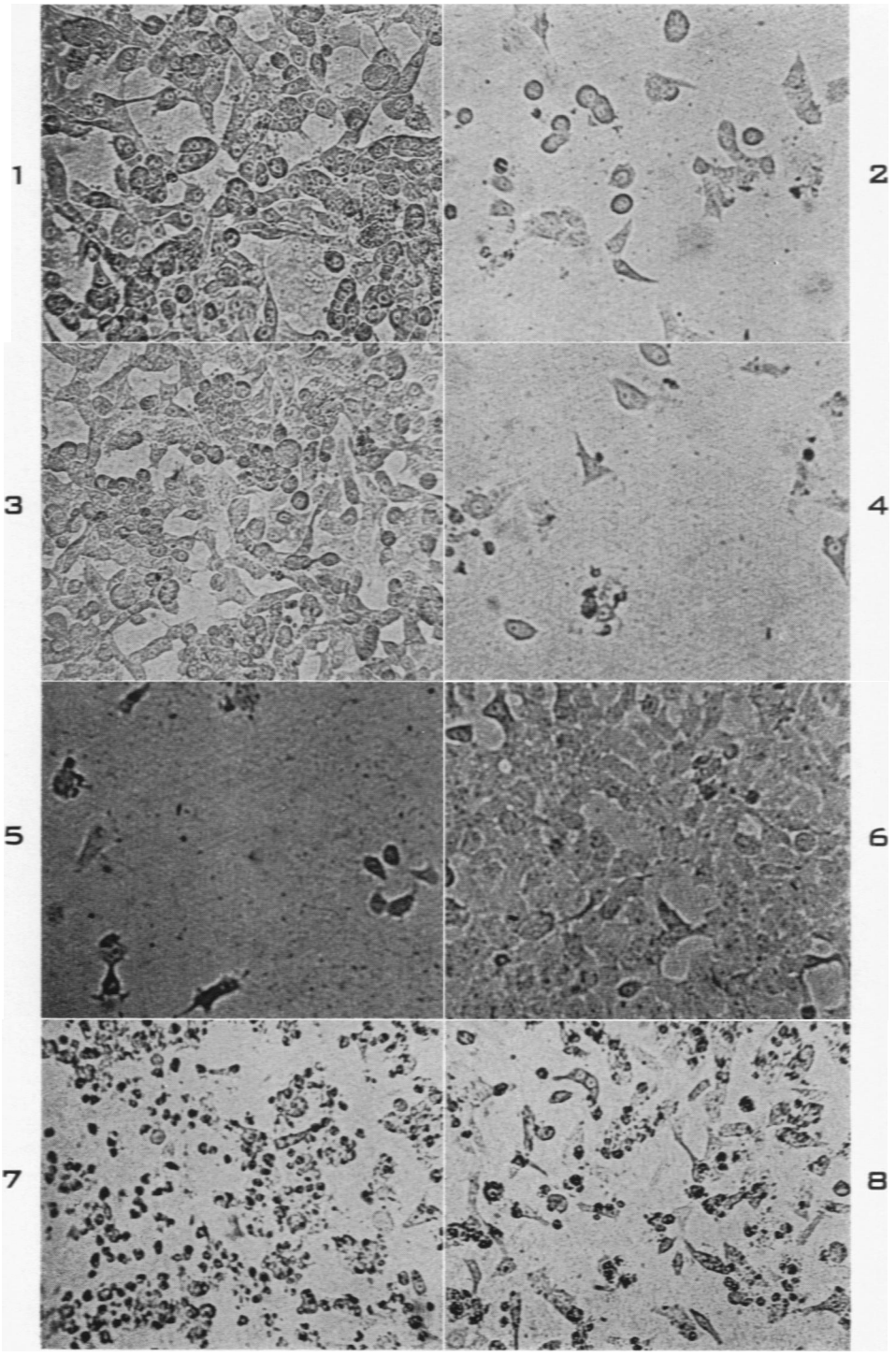
FIG. 3. Normal strain HeLa cells in an uninoculated control culture, photographed on the same day as the culture shown in Figure 2. × 150.

FIG. 4. Strain HeLa cells, photographed 4 days after inoculation of Eastern equine encephalomyelitis virus to demonstrate that a few normal appearing cells remain, although extensive destruction has occurred. × 150.

FIG. 5. The cytologic effect of Western equine encephalomyelitis virus for strain HeLa cells as seen 1 day after the inoculation of virus. × 150.

FIG. 6. Normal appearing strain HeLa cells protected by specific antibody from the cytopathogenic effect of Western equine encephalomyelitis virus. × 150.

FIGS. 7 and 8. Destruction of strain HeLa cells by West Nile virus, photographed 6 days after the inoculation of virus. × 125.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