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BIOLOGIC AND ANTIGENIC VARIATION OF JAPANESE ENCEPHALITIS VIRUSES*

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Indexing Words

Japanese encephalitis virus; variation; antigenicity; tissue culture

Shigeru MAKINO, Nobuya FUJITA, Hideo AOKI, Manabu TAKEHARA and Susumu HOTTA. *Biologic and Antigenic Variation of Japanese Encephalitis Viruses.* Kobe J. Med. Sci. 17, 75-84, June 1971——One Japanese encephalitis (JE) virus strain, designated as MH30, was directly isolated from *Culex tritaeniorhyncus* mosquitoes by use of cell culture, without being passed in the mouse brain, and was serially passed through primary hamster kidney (HK) cells cultivated at 30°C.

After about 40 cell culture passages, the strain became stable in certain of its biologic properties. When compared with Gl strain, a JE virus strain of human origin which was also passed through HK cells under the same condition, the MH30 strain was characterized by higher infectivity for HK cells, lower pathogenicity for mice both by the intracerebral and intraperitoneal routes of injection and higher homogeneity in fractionation on DEAE cellulose column chromatography.

The MH30 strain was identified as JE virus by cross neutralization tests with Gl strain virus. However, relative potency of anti-MH30 serum against Gl virus was higher than that of anti-Gl serum against MH30 virus. Apparently there was a quantitative difference between MH30 and Gl strains in antigenicity.

INTRODUCTION

The variation of viruses has been investigated from several vantage points, from basic problems such as bacteriophage mutation to practical aspects such as development of live vaccines. As to the latter, particular attention has been paid to the correlation of the pathogenicity of viruses and their genetic marker(s) controlling biologic and immunologic features as such.

Decrease of pathogenicity of JE virus by passages through tissue culture has been demonstrated by various investigators (Bhatt and Work, 1957; Lee et al, 1958; Oya et al, 1960; Rosenberger and Shaw, 1961; Hotta et al, 1961; Inoue et al, 1961; Shimizu, 1963) but was first demonstrated by Kawakita (1939) using chick embryonic tissues. Attenuation of pathogenicity of JE virus by serial cultivation in one-day-old chicken eggs has also been reported (Okuno, 1959). However, mechanism(s) underlying such changes have not yet been fully clarified. This paper describes the passage

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of JE virus strains isolated in Japan through tissue culture and properties of the resulting viruses, including *in vivo* and *in vitro* infectivity, chromatographic fractionation, and immunologic reactivity.

MATERIALS AND METHODS

Virus

A JE virus strain, designated MH30 (mosquito-hamster 30°C), was directly isolated into porcine kidney stable cells (PS-15) (Inoue and Yamada, 1964) from an emulsion of a pool of *Culex tritaeniorhyncus* mosquitoes* and then passed serially in HK cell cultures.

Two JE viruses of human origin, Gl strain (250th mouse brain passage) and JaTH strain (first mouse brain passage), were also used.

Cell Culture

Techniques for preparing HK cells were essentially the same as previously described by Ishiga *et al* (1960). The medium used consisted of 10 parts of inactivated calf serum (free of anti-JE virus antibodies) and 10 parts of 5% lactalbumin hydrolysate - 1% yeast extract in Hanks' balanced salt solution (BSS) supplemented with 100u/ml penicillin and 100γ/ml streptomycin. The temperature for initial cell growth was 37°C and that for incubation after virus inoculation was 30°C

Serial Passages and Titration of Virus

The JE virus strains were serially passed through HK cell cultures by the following procedures: The infected fluid was inoculated into 3 cell culture tubes; multiplicity of infection was 10 to 100. After virus adsorption at 30°C for 90 minutes, the cells were incubated at 30°C for 3 to 4 days until characteristic cytopatic effect was observed. The culture fluid was harvested, centrifuged at a low speed (800 $\times$ *g*), and the supernatant fluid inoculated onto fresh cells. Repeated passage of virus was carried out in this manner.

Viral infectivity was titrated in weanling mice weighing 7-8 g or in HK cells. Mouse intracerebral LD_{50} per ml ($MICLD_{50}$), mouse intraperitoneal LD_{50} per ml ($MIPLD_{50}$) and HK 50% tissue culture infective dose per ml ($HKTCID_{50}$) were determined by the method of Reed and Muench (1938), employing 5 mice or 5 cell culture tubes for each 10-fold dilution. Infectivity of the serially passed viruses was usually determined once every fifth passage.

Plaque Formation in BHK-21 Cell Culture

BHK-21 cells, clone 13 (Stoker and Macpherson, 1964) were cultivated in

* The first passage infected PS culture fluid was kindly supplied by Dr. Y. Matsuyama and his staff of the Public Health Institute of Kyoto City.

Eagle's minimum essential medium (MEM) (Eagle, 1959) supplemented with 10% calf serum free of anti-JE antibodies. After 3 or 4 days incubation at 37°C, the cell monolayers were washed with BSS. One-tenth ml/bottle of virus was inoculated onto the cell monolayer after appropriately diluting the virus in phosphate buffer (pH 7.0) supplemented with 0.1% bovine plasma albumin (Fraction V, Difco, Detroit). The cells were held at room temperature for 2 hours with occasional agitation to aid virus adsorption. The cells were overlaid with MEM supplemented with 3% calf serum and 1% methyl cellulose (4000 cps), and then incubated at 37°C for 5 days. The cells were stained with crystal violet to locate the plaques to determine the plaque forming units (PFU) / ml of inoculum.

Purification of Virus

The viruses were partially purified by a modification of the method described by Matsumura *et al.* (1967): (1) Cell debris was removed from infected supernatant fluid or mouse brain homogenates by centrifugation at $1,000 \times g$ for 15 minutes at 4°C; (2) the supernatant fluid was removed and mixed with protamine sulfate using a final concentration of 1 mg/ml and held at 4°C for 45 minutes with occasional agitation; (3) the mixture was centrifuged $2,500 \times g$ for 15 minutes and the supernatant used as purified material.

DEAE Cellulose Column Chromatography

Five ml of the purified virus suspension was added into a column (10×1.5cm) containing a preconditioned DEAE cellulose*. The virus was eluted stepwise with phosphate buffer (pH 7.2) containing increasing concentrations of NaCl (0.1 M increments), from 0–0.7 M NaCl using 4 ml volumes for each elution step. Usually 16 fractions, 2 ml each, were collected. The infectivity of each fraction was measured by a plaque assay in BHK-21 cells. Hemagglutinin activity (HA) was also measured by the method of Clarke and Casals (1958). Recovery of infectivity and HA after elution was usually 90 to 95%

Virus Neutralization

Viral antisera were prepared by injecting 3 male rabbits with the MH30 or Gl strain virus intravenously. Six injections were given at one week intervals using virus inocula in the range of 10^5 to 10^6 $HKTCID_{50}$. One week after the last injection, the animals were exanguinated and the serum was separated. The sera were inactivated by heating at 56°C for 30 minutes and stored at −80°C.

(1) Plaque reduction: Sera were diluted in two-fold increments. Equal volumes of virus (200 PFU/0.1 ml) and the diluted serum were mixed and held at 37°C for 60 minutes. The virus-serum mixture (0.1 ml) was added to each of 4 replicate bottles of BHK-21 cells for PFU determination. Arithmetic means of the plaque numbers were calculated. The same procedures were carried out in parallel using normal rabbit serum. Fifty percent effective dose (dilution of immune serum

* Pharmacia Fine Chem., Piscataway, New Jersey, 0.48 meq/ml

producing 50% reduction in number of plaques, ED_{50}) was estimated by probit analysis (Finney, 1952). Relative potency (RP), i. e., ratio of heterologous to homologous ED_{50}'s (multiplied by 100) was also calculated.

(2) Neutralization kinetics: Two ml of virus ($10^{6.8}$PFU/ml) and 2 ml of appropriately diluted antiserum were mixed and incubated at 37°C. An aliquot of the mixture (0.1 ml) was removed at 0, 10, 20, 40 and 60 minutes after incubation and mixed with 9.9 ml of precooled 0.1% bovine plasma albumin in phosphate buffer (pH 7.0). The virus was further diluted 10-fold and the residual infectivity titrated in BHK-21 cells.

RESULTS

Infectivity Changes after Serial Passage in HK Cells

Infectivity of the virus was determined in mice and in HK cells during the course of serial passage in cell culture (Fig. 1).

Gl strain virus retained high $MICLD_{50}$ and $HKTCID_{50}$ (Fig. 1A). However, the $MIPLD_{50}$ decreased during the cell culture passage. JaTH strain virus showed

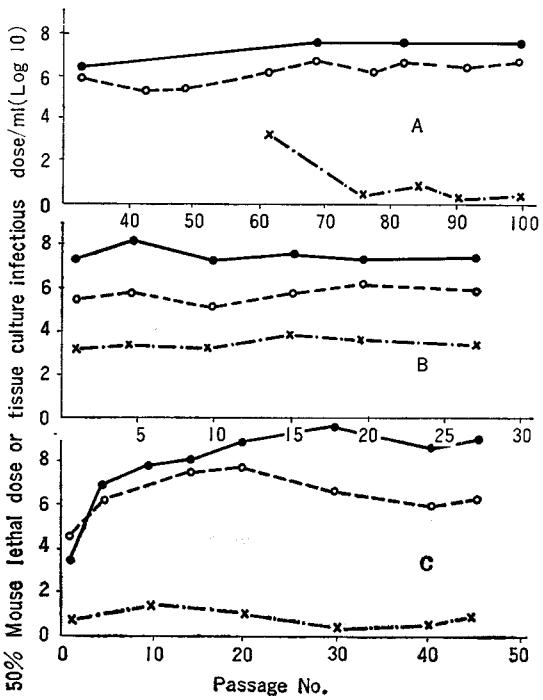

Fig. 1. Passage of Japanese encephalitis (JE) virus in primary hamster kidney cells; (A) JE virus, Gl strain, (B) JaTH strain and (C) MH30 strain. Graph shows (• — •) 50% tissue culture infectious close in primary hamster kidney cells, (○ --- ○) 50% mouse intracranial lethal dose and (×—•—×) 50% mouse intraperitoneal lethal dose.

no change in its infectivity in mouse and in cell culture (Fig. 1B). In contrast to these observations, the $MICLD_{50}$ of MH30 strain decreased after the 20 passage in HK cells while the infectivity in HK cells showed an increase from the 5–30 passage (Fig. 1C). The difference in the $MICLD_{50}$ and the $HKTCID_{50}$ of MH30 was about $10^{2.5}$ by the 40th passage. The $MIPLD_{50}$ of the same strain was relatively low throughout the serial passage. The low pathogenicity in mice was not changed after the virus was further passed through the mouse brain.

DEAE Chromatographic Elution Patterns

Illustration of elution patterns of Gl and MH30 strains on DEAE chromatographic columns is shown in Figures 2 and 3.

Gl strain showed two major peaks elution with 0–0.1M NaCl and elution with 0.5–0.7 M NaCl, whereas the MH30 strain had one major peak (elution with 0–0.2 M NaCl). In other words, the Gl strain was heterogeneous whereas the MH30 strain appeared to be homogenous chromatographically. The elution pattern of Gl strain after 5 further passages through MK cell cultures resembled that of MH30. The MH30 strain showed no further chromatographic change after being passed in mouse brain.

Virus Neutralization

Examples of cross neutralization results using the plaque reduction method are shown in Figure 4 and Table 1.

The percentage of surviving virus (plotted as probit values) is proportional to the dilution of serum (reciprocal of $\log_2$). The slopes are essentially the same in all the homologous and heterologous virus neutralizations. However, relative potency fo anti-MH30 serum against Gl virus is higher (40.9) than that of anti-Gl serum

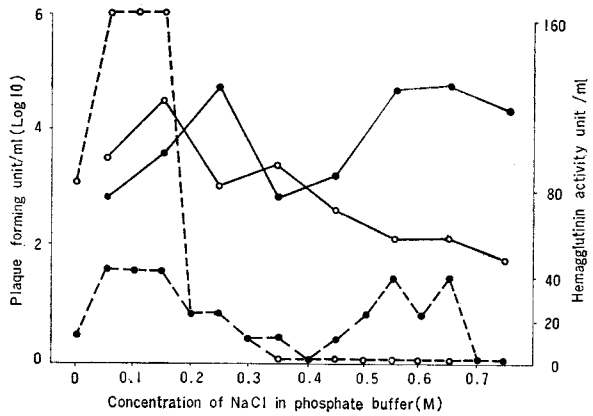

Fig. 2. Elution pattern of Japanese encephalitis virus, Gl strain on DEAE column. (•) The virus after being passed through mouse brain 250 times, (○) the same virus after further being passed through primary hamster kidney cells 5 times. (—) Plaque forming units/ml in BHK_{21} cells. (···) Hemagglutinin activity/ml.

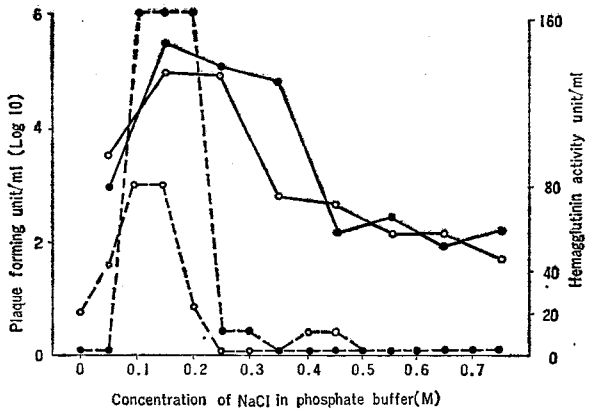

Fig. 3. Elution pattern of Japanese encephalitis virus, MH30 strain on DEAE column. (•) The virus after being passed through primary hamster kidney cells 40 times. (∘) the same virus after further being passed through mouse brain 5 times. (—) Plaque forming unit/ml in BHK_{21} cells. (---) Hemagglutinin activity/ml.

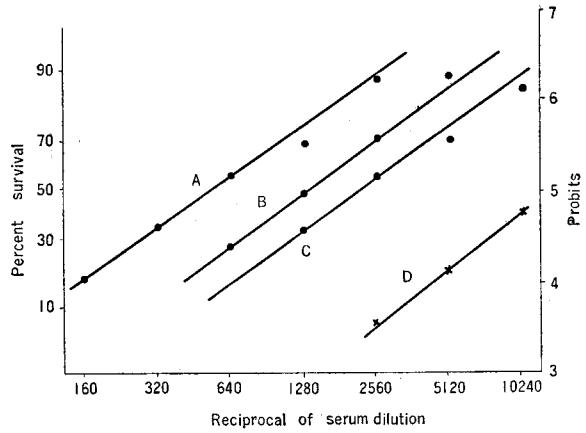

Fig. 4. Plaque reduction neutralization tests of Japanese encephalitis virus. (A) Gl strain-anti-MH30 antiserum, (B) MH30 strain-anti-MH30 antiserum, (C) MH30 strain-anti-Gl antiserum and (D) Gl strain-anti-Gl antiserum. Virus and antiserum of each dilution were mixed and held at 37°C for 60 minutes. Surviving virus was titrated by plaque assay in BHK_{21} cells.

Table 1. Plaque reduction neutralization test of Japanese encephalitis virus, Gl strain and MH30 strain

Antiserum	Strain	Preciprocal of ED_{50}*	95% CL+ of ED_{50}	RP‡	95% CL of RP
Anti MH30	MH30	1390	1165–1650	100.0	33.4–50.0
	Gl	569	520– 620	40.9	
Anti Gl	Gl	14080	12580–17820	100.0	15.1–23.8
	MH30	2661	2267– 2961	18.9	
Control	MH30	24	21–28		
	Gl	14	12–16		

* Serum dilution producing 50% reduction in number of plaques

+ Confidence limit

‡ Relative potency of the serum against heterologous virus to homologous one (multiplied by 100)

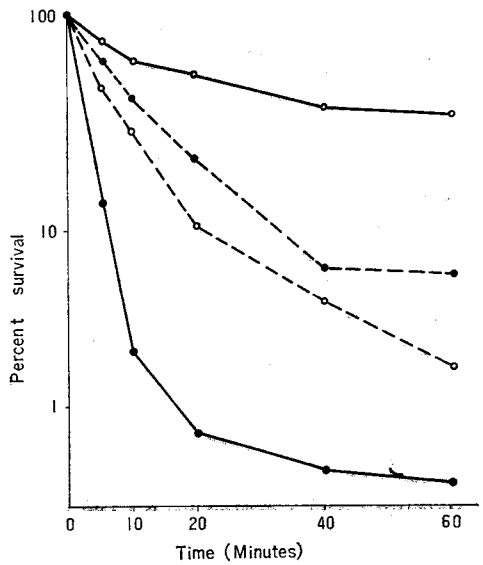

Fig. 5. Neutralization kinetics of Japanese encephalitis virus. (∘ — ∘) MH30 strain-anti-Gl antiserum, (• ··· •) Gl strain-anti-MH-30 antiserum, (∘ ··· ∘) MH30 strain-anti-HH30 strain and (• — •) Gl strain-anti-Gl antiserum. Virus and antiserum were mixed and held at 37°C. An aliquot (0.1 ml) of the mixture was removed after each incubation period and mixed with 9.9 ml of precooled 0.1% bovine plasma albumin in phosphate buffer. The virus was further diluted 10-fold and titrated by plaque assay in BHK_{21} cells.

against MH30 virus (18.9) (Table 1).

The result of neutralization kinetic experiments are illustrated in Figure 5. The rate of neutralization of Gl strain by anti-MH30 serum was more rapid and to a higher degree than that of MH30 strain by anti-Gl serum.

DISCUSSION

Rohitayodhin and Hammon (1962) demonstrated that changes of animal pathogenicity during tissue culture passage were apparently variable among virus strains of different origins; i. e., Nakayama strain passed in mice retained its mouse pathogenicity after serial passage in tissue culture, whereas another strain of mosquito origin (OCT-541) showed distinct attenuation using the same method of cell passage. In the present study it was clearly shown that mouse and cell culture infectivity and the variation in infectivity during serial cell passage differed among the virus strains tested (Fig. 1). Henderson *et al* (1967 a, b), using Eastern and Western equine encephalitis viruses, demonstrated the presence of virus subpopulations which reached equilibrium after serial cell culture passage by their cross neutralization criteria. Selection and ultimate accumulation of certain subpopulations could show the emergence of variants. A similar explanation could be ascribed to the MH30 strain.

The chromatographic pattern of MH30 strain was homogeneous whereas the Gl strain was heterogeneous. The Gl strain data were compatible with the results of other wokers. Heterogeneous properties of selected mouse-passaged strains of JE virus have been described by previous investigators using column chromatography (Takehara, 1965; Gaidamovich and Myou, 1966) or density gradient centrifugation (Igarashi et al, 1963, Kitaoka and Nishimura, 1965) techniques. The results of the data suggest that MH30 strain virus showed a broader "antigenic specturm" than does Gl strain virus (Fig. 4-5). From a practical point of view, MH30 strain may be more suitable as an immunizing virus than the Gl strain.

The results so far available suggest that there are some genetic difference(s) among JE virus strains. Problems of whether, or how, such genetic features can be correlated with phenotypic expressions, for example, as demonstrated in pathogenicity tests, deserve further research. This is not only interesting from a basic point of view relative to the nature of virus, but also is important for clinical and public health problems. It is well known that patients with a severe JE syndrome are relatively limited in spite of wide distribution of specific antibodies among humans. One possible explanation for this fact may be the change in the virus population during passage in the mosquito which selects for a virus of low human pathogenicity. Such variants may be useful for development of live attenuated vaccines.

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