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Growth Patterns of Six Strains of Japanese Encephalitis Virus

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Abstract: Growths of 6 strains of Japanese encephalitis (JE) virus were studied in mosquito (C6/36) and baby hamster kidney (BHK21) cell cultures at 4 different temperatures. At 28℃, 3 strains (Nakayama, Beijing−1, JaOArS982) grew slower than other 3 strains (JaGAr01, JaOH0566, ML17) in C6/36 cells although their infectivity increased from 1 day after infection. On the other hand, infectivity of all strains increased from 2 days after infection in BHK21 cells. At 33 and 37℃, growth of JaOArS982 strain in C6/36 cells was shut-off 2 and 1 day after infection, respectively, while growths of all strains in BHK21 cells were shut-off 2 days after infection. At 39℃, growths of all strains were shut-off 1 day after infection in both cell lines, and infectivity of Nakayama strain showed little increase in C6/36 cells. The results indicated some host cell factor(s) regulating replication of JE virus, especially in C6/36.

Key words: Growth pattern, Japanese encephalitis, Infectivity

INTRODUCTION

Japanese encephalitis (JE) virus is a member of the family Flaviviridae (Westaway *et al.*, 1985), which was formerly classified as mosquito-borne group B arbovirus (Clarke and Casals, 1965; Fiedman, 1968). JE is a serious public health problem with significant mortality and grave sequelae in many Asian countries (Miles, 1960; Umenai *et al.*, 1985). Since the first isolation in 1935 in Japan, many strains of JE virus have been isolated from mosquitoes, birds, swine, horses, and humen. In spite of many investigations of different aspects of JE virus, detailed knowledge on its growth patterns in mosquito and mammalian cell lines using different strains at various temperatures is rather limited.

This paper describes growth patterns of 6 strains of JE virus at 4 temperatures in order to pinpoint the host-virus-temperature relationship in JE virus production.

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MATERIALS AND METHODS

Cell: Aedes albopictus, clone C6/36 cells (Igarashi, 1978) were grown at 28℃ with cell growth medium of Eagle's minimal essential medium supplemented with 0.2mM each of non-essential amino acids (Eagle, 1959) and 10% heat-inactivated fetal calf serum (FCS). BHK21 cells were grown with the same medium but at 37℃.

Virus: Six strains of JE virus were used in this study: Nakayama Yoken, Beijing−1, JaGAr01, JaOH0566, ML17 and JaOArS982, and their origins were described previously (Hori *et al.,* 1986a; b). Confluent cultures of C6/36 cells in 750cm^3 Roux bottles were inoculated with each strain of JE virus. After 2 hours of adsorption, the cells were fed with 40 ml/bottle of maintenance medium (cell growth medium from which FCS concentration was reduced to 2%) and incubated at 28℃. After 3 days, infected culture fluids were harvested and stored in aliquots at −70℃ as seed virus and their infectivities were determined.

Virus growth experiment: Growth medium was removed by aspiration from C6/36 or BHK21 cells on 24 well semimicroplates (Falcon 3047, USA), and 0.1ml of seed virus was inoculated into each well at appropriate dilution to input multiplicity of infection of 0.1 PFU/cell. Virus adsorption was carried out for 2 hours with spreading the inoculum every 30 minutes. Then, residual virus was removed and 1ml of maintenance medium was added to each well and the cells were incubated in 5% CO_2−atmosphere at respective temperatures. Aliquots of infected culture fluid was harvested every day from duplicate wells and virus infectivity in pooled specimens was titrated.

Infectivity titration of the virus: The method was modifed from that described by Hashimoto *et al.* (1971). Specimens were serially diluted in 10-fold steps with 5% FCS in Eagle's medium and inoculated to BHK21 cells on semimicroplates after their growth medium was removed, using 0.1 ml/well and duplicate wells for each dilution. After 2 hours of adsorption at 37℃ by spreading the inoculum every 30 minutes, residual virus was removed and the cells were covered with 1 ml/well of overlay medium (1.5% methyl cellulose and 1% FCS in Eagle's medium) and incubated at 37℃ in 5% CO_2−atmosphere for 5 days. The overlay medium was removed by rinsing with phosphate buffered saline with calcium and magnesium. The cells were fixed with cold methanol for 30 minutes and stained with 0.1% Trypan blue at room temperature for 1 hour to reveal the plaques. Infectivity of the virus was recorded as plaque forming unit (PFU) per ml.

RESULTS

The growth curve of 6 different strains of JE virus in C6/36 cells and BHK21 cells at 4 different temperatures were shown in Fig. 1. At 28℃, 3 strains of JE virus (Nakayama, Beijing−1, JaOArS982) grew slower than 3 other strains (JaGAr01, JaOH0566, ML17) in C6/36 cells, and the difference was remarkable 1 day after infection, when all

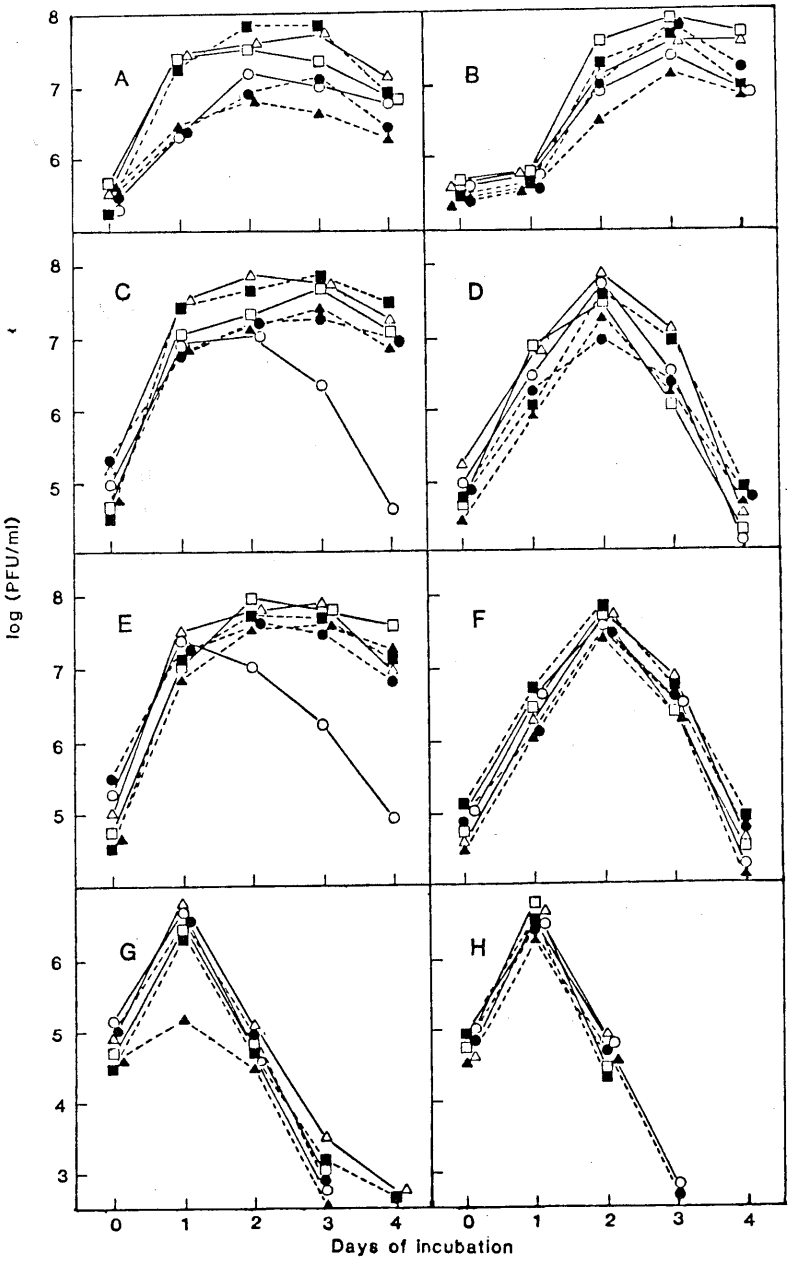

Fig. 1. Growth curve of 6 strains of JE virus in C6/36 cells and BHK21 cells at 4 temperatures.
Each of the 6 strains of JE virus, Nakayama (▲----▲), Beijing-1 (●----●), JaGA01 (□——□), JaOH0566 (△——△), ML17 (■----■), and JaOArS982 (○——○), was inoculated to C6/36 (A, C, E, and G) or BHK21 (B, D, F, and H) cells, and incubated at 28℃ (A and B), 33℃ (C and D), 37℃ (E and F), and 39℃ (G and H), to assay virus infectivity in the infected fluids by plaque titrtion on BHK21 cells at 37℃. Infectivity was undetectable for Nakayama, Beijing-1, JaGAr01, and JaOArS982 strains 4 days after infection in C6/36 cells at 39℃ (G), for Nakayama, JaGAr01, JaOH0566 and ML17 strains 3 days after infection, and for all strains 4 days after infection in BHK21 cells at 39℃ (H).

strains showed titer increase. While in BHK21 cells almost no titer increase was observed 1 day after infection for all strains, and their titers increased 2−3 days after infection. Difference among virus titers of various strains in BHK21 cells 3 days after infection was not so remarkable, although JaGAr01 showed the highest and Nagayama the lowest titer, respectively, 2 days after infection.

At 33℃, all strains grew up faster than at 28℃ both in C6/36 cells and BHK21 cells with similar rates up to 2 days after infection. But after 2 days of incubation, virus production was shut-off for JaOArS982 strain in C6/36 cells and all strains in BHK21 cells, and infectivity of 5 remaining strains in C6/36 cells did not change significantly.

Similar findings were obtained at 37℃, but shut-off of JaOArS982 strain in C6/36 cells took place earlier, that is 1 day after infection.

At 39℃, all strains showed sharp decline in their titers after 1 day's increase in both cell lines, and infectivity of Nakayama strain increased less than other strains in C6/36 cells. Such strain difference was not observed in infected BHK21 cells.

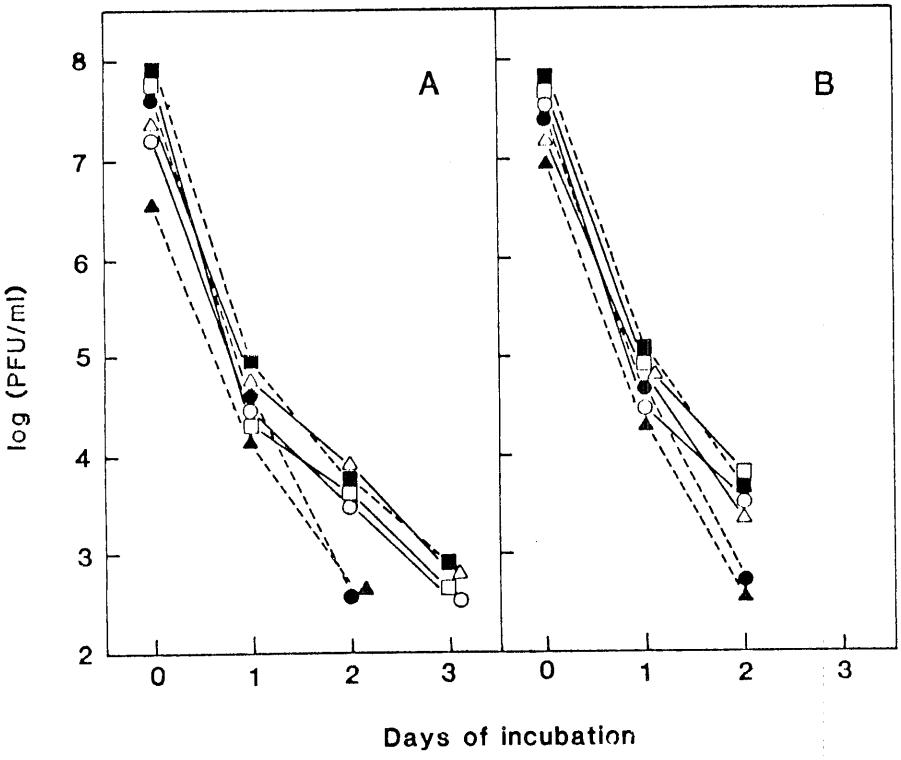

Fig. 2. Heat-stability of JE virus infectivity
Each of the 6 strains of JE virus, Nakayama (▲----▲), Beijing−1 (●----●), JaGA01 (□——□), JaOH0566 (△——△), ML17 (■----■), and JaOArS982 (○——○), was inoculated at 37℃ (A), and 39℃ (B), and specimens were sampled every day to assay their infectivities on BHK21 cells at 37℃. Infectivity was undetectable for Nakayama and Beijing-1 strains 3 days after incubation at 37℃ (A), and for all strains 3 days after incubation at 39℃ (B).

In order to explain the different growth curves for 6 strains of JE virus, heat-stability experiments were carried out. Each strain of seed virus was incubated at 37 and 39℃ and specimens were titrated for their infectivity every day until 4 days of incubation. The results in Fig. 2 showed that at 37℃ infectivity of all strains decreased 2−3 logs within a day, and Nakayama and Beijing−1 strains were more rapidly inactivated than other strains after 2 days of incubation and their infectivities became undetectable on the 3rd day. While, other strains were inactivated slower after 2 days of incubation and low levels of infectivities were still detectable evan after 3 days of incubation. After 4 days of incubation, infectivity was not detectable for all strains.

At 39℃, inactivation rate was almost similar to that at 37℃ for the 1st day of incubation and Nakayama and Beijing−1 strains were slightly more rapidly inactivated than other strains during subsequent days of incubation. Infectivity became undetectable after 3 days of incubation for all strains.

DISCUSSION

Recently Igarashi and Srivastava (1988) reported that growth of some flaviviruses is dependent on the combination of virus and host cells. In the present study, we found some difference in the growth patterns of JE virus strains, particularly in C6/36 cells, although their basic growth patterns were essentially similar to all the strains in BHK21 cells. Since no significant difference was observed among JE virus strains in their heat-stability and all strains showed similar growth rates in BHK21 cells, the shut-off of JaOArS982 strain in C6/36 cells at 33 and 37℃ after initial growth phase cannot be explained by its reduced heat-stability. Therefore, the shut-off phenomenon could be the result of reduced virus production, either by regulation on the processes of viral RNA replication, assembly and maturation which depend on some host cell factor(s), or by viral cytopathic effect which significantly damages host macromolecular syntheses required for the viral production. These possibilities should be investigated by further study.

ML17 is an attenuated vaccine strain for swine and was obtained from JaOH0566 strain by serial passages and plaque purification in cell cultures at reduced temperatures, and has altered biological characteristics such as reduced swine viremia, reduced mouse neurovirulence, and reduced infectivity to vector mosquitoes, *Culex tritaeniorhynchus* (Yoshida *et al.*, 1981). It is therefore unexpected that no significant difference was found between JaOH0566 and ML17 strains in their growth patterns in C6/36 cells and BHK21 cells at different temperatures. The result shows that *in vitro* markers do not always parallel to *in vivo* markers.

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