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In Vitro Difference between an Attenuated ML－17 and its Parental Virulent JaOH0566 Strain of Japanese Encephalitis Virus

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Abstract: Two Japanese encephalitis (JE) virus strains, namely a virulent JaOH0556 and an attenuated ML－17 derived from it were studied to investigate their *in vitro* phenotypic differences. When both strains were concentrated by polyethylene glycol (PEG) 6000 precipitation, infectivity of the ML－17 was recovered only 4.94% compared to 42.21% for the parental JaOH0566 strain, and the virion peak was very low for the ML－17 in contrast to a sharp peak for the JaOH0566 in sucrose density gradient centrifugation. When both strains were incubated with sucrose solutions, the ML－17 was slightly more labile than the JaOH0566 at high concentration of sucrose. These findings may indicate difference in the virion structure of both strains.
Key words: Attenuation, Polyethylene glycol, Japanese encephalitis virus

INTRODUCTION

Japanese encephalitis (JE) virus is a member of the family Flaviviridae (Westaway *et al.,* 1985), and also a mosquito－borne arbovirus (Berge, 1975) with major vertebrate amplifier of swine (Scherer *et al.*, 1959; Konno *et al.*, 1966) and vector mosquitoes of *Culex tritaeniorhynchus* (Mitamura *et al.*, 1938; Buescher *et al.*, 1959). JE has been counted among important public health problems in East, Southeast and South Asia causing severe encephalitis in humans and horses with significant mortality and grave sequelae and also abortion of pregnant swine (Miles, 1960; Umenai *et al.*, 1985; Monath, 1988). Although several strains isolated from different geographic areas showed different genomic structures by RNA oligonucleotide fingerprint analysis (Hori, 1986), phenotypic and genotypic

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differences between virulent and attenuated JE virus strains have not well been documented. An attenuated live vaccine strain, ML−17, was derived from a virulent JaOH0566 strain isolated from the brain of a fatal JE case through serial passages and repeated plaque cloning (Yoshida *et al.,* 1981). The vaccine strain showed altered biological markers such as reduced virulence in experimental animals and reduced growth in *Culex tritaeniorhynchus,* and was regarded as an appropriate live vaccine to immunize swine to prevent viremia and amplification of JE virus in nature. However, this attenuated strain has not been characterized for its *in vitro* markers. In order to analyze the ML−17 strain, repeated attempts to purify the virion could not yield sufficient amount of the purified virion, although it showed similar growth pattern to its parental strain in cultured cells (Shameem *et al.,* 1988). These findings suggested that the virion of ML−17 strain could be less stable than its parental strain. In this paper, we describe that ML−17 strain is more labile than its parental JaOH0566 strain for polyethylene glycol (PEG) treatment which has been used routinely for virus purification.

Materials and Methods

Cells: A clone C6/36 of *Aedes albopictus* cells (Igarashi, 1978) was used for virus growth. The cells were grown at 28℃ in spinner bottles in suspension with 1 liter/bottle (Morita and Igarashi, 1989) with Eagle's medium supplemented with 0.2 mM each of nonessential amino acids (Eagle, 1959) and 10% heat−inactivated fetal calf serum (FCS). BHK21 cells were used for titration of the virus infectivity and were grown at 37℃ with the same medium as C6/36 cells. In the maintenance medium after virus inoculation, FCS concentration was reduced to 2%.

Virus: Both JaOH0566 and ML−17 strains were obtained from Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University (Hori *et al.,* 1986), and seed viruses were prepared in stational culture of C6/36 cells.

Immunoperoxidase staining of the virus: Virus infectivity was assayed by immunoperoxidase staining with slight modification from that of Okuno *et al.,* (1985). After inoculation of virus specimens serially diluted by 5% FCS in Eagle's medium and 2 hr adsorption at 37℃, the cells of 96−well microplates were covered with 1% Tragacanth gum in the maintenance medium and incubated at 37℃ in 5% CO_2 for 30 hr. Then the cells were fixed by 5% formaldehyde in phosphate buffered saline (PBS), pH 7.4, at room temperature (RT) for 20 min, and the cell membrane was permealized by 1% Nonidet P−40 in PBS for 20 min at RT. After reactions with anti−JE mouse serum and peroxidase−conjugated anti−mouse IgG rabbit IgG (DAKO, Denmark), Peroxidase reaction was carried out with 1.2 mg/ml of 3,3'−diaminobezidine 4 HCI and 0.01% H_2O_2 in PBS at RT for 20 min. Foci of infected cells were counted by stereoscope and infectivity was expressed as focus forming unit (FFU)/ml.

Virus concentration and purification: The procedure was carried out according to Srivastava

et al., (1987). When C6/36 cells grew up to about 10^6 cells/ml, microcarrier (Cytodex−1, Pharmacia, Sweden) was added to 2 mg/ml to attach the cells on it. After 12 hr, the cells on microcarrier were sedimented by standing the bottles on the bench and culture fluid was removed by decantation. Forty ml of seed virus was added to the sedimented cells in each bottle and virus adsorption was carried out at RT for 2 hr. Then the cells were supplied by 750 ml/bottle of the maintenance medium and spinner culture was continued. After 2 days of virus inoculation, infected culture fluid was harvested and the cells were supplied by fresh maintenance medium and infected fluid was harvested every alternative day altogether 3 times. Infected culture fluid (S1) was passed through No. 2 filter paper (Advantec−Toyo, Japan), and centrifuged at 10,000g for 15 min at 4℃ to get the supernatant (S2). Polyethylene glycol (PEG) 6000 (Wako Pure Chemical Industries, Japan) and NaCl were added to S2 to final concentrations of 6% and 0.5 M respectively. When they were completely dissolved, the mixture was centrifuged at 10,000g for 30 min at 4℃ and the supernatant (S3) was removed. The sediment was resuspended in STE buffer (0.1 M NaCl, 0.01 M Tris−HCl, 0.001 M EDTA, pH 7.4) using 24 ml for each harvest. The suspension (S4) was centrifuged at 20,000g for 15 min and the supernatant (S5) was layered on 10 ml of 30−45% linear sucrose gradient and 2 ml of 15% sucrose in STE buffer. The specimen was centrifuged at 20,000 rpm for 20 hr at 4℃ using SW28 rotor of a Beckman model L8−80M ultracentrifuge. Fractions were collected by ISCO density gradient fractionator model 640 recording OD_{254}.

Effect of sucrose on the infectivity of JE virus: The specimens S5 of both ML−17 and JaOH0566 strains were diluted 1:10 with various concetration of sucrose solutions in STE buffer to get the final sucrose concentration of 0%, 15%,25%, 35% and 45%, and the mixtures were incubated at 4℃ for 2 hr. Infectivity of these solutions was assayed as described above and compared with the titer of S5 which was diluted 1:10 by the virus diluent and incubated in parallel with the specimen of sucrose.

RESULTS

Effect of PEG on the infectivity of ML−17 and JaOH0566 strains

Table 1 shows the infectivity of both ML−17 and JaOH0566 strains at various steps of virus purification. Although virus titer was similar for both strains in the starting material (S1) and after filtration and clarification (S2), recovery of the infectivity in S4 was 42.21% for JaOH0566 in contrast to 4.94% for the ML−17. This difference cannot be explained by poor precipitation of the ML−17 because the virus titer in S3 was 3.9% of the original specimen for JaOH0566 in contrast to 0.03% for ML−17 strain. The results rather suggest that the virion of ML−17 is less stable than that of JaOH0566 for PEG treatment.

Figure 1 shows a sharp peak corresponding to the complete virion of the JaOH0566 strain (upper panel) in contrast to a very low peak of the ML−17 strain (lower panel) in

Table 1. Recovery of JaOH0566 and ML-17 strains by polyethylene glycol precipitation

Specimens*	JaOH0566		ML-17	
	Total infectivity in FFU	Recovery %	Total infectivity in FFU	Recovery %
S1	1.40×10^{11}	100.00	8.00×10^{10}	100.00
S2	1.19×10^{11}	85.00	6.87×10^{10}	85.87
S3	5.46×10^{9}	3.90	2.15×10^{7}	0.03
S4	5.91×10^{10}	42.21	3.95×10^{9}	4.94
S5	5.48×10^{10}	39.14	3.42×10^{9}	4.27

* S1=Original infected fluids (750 ml); S2=Supernatant of high speed centrifugation after filtration through filter paper; S3=Supernatant after PEG treatment of S2; S4=PEG precipitated S2 and resuspended in STE buffer (24 ml); S5=Supernatant of S4 after high speed centrifugation. Figures represent average of 3 independent experiments.

sucrose gradient sedimentation of specimen S5. The result may suggest that the virion of ML−17 was more easily disintegrated than that of JaOH0566 during sucrose gradient sedimentation.

Effect of sucrose on the infectivity of ML−17 and JaOH0566 strains

In order to test the possibility that ML−17 strain is more easily disintegrated than its parental strain during sucrose gradient sedimentation, S5 specimens after PEG precipition of both strains were incubated with various concentration of sucrose solutions at 4℃ for 2 hr and the infectivity was titrated. Table 2 shows that incubation with sucrose solutions did not show very much apparent inactivation of the ML−17 than the JaOH0566, but at high concentration of sucrose (35% and 45%) recovery of the ML−17 was slightly less than that of the JaOH0566. Both strains were inactivated a little in STE buffer compared with the original S5 specimen in virus diluent with 5% FCS in Eagle's medium.

Table 2. Effect of sucrose on the infectivity of JaOH0566 and ML-17 strains after PEG treatment

Sucrose Concentration in STE (%)	JaOH0566		ML-17	
	Infectivity in FFU/ml	Recovery %	Infectivity in FFU/ml	Recovery %
0	1.61×10^{9}	70.6	0.97×10^{8}	67.8
15	1.55×10^{9}	68.0	1.11×10^{8}	77.6
25	1.63×10^{9}	71.5	1.15×10^{8}	80.4
35	1.69×10^{9}	74.1	0.87×10^{8}	60.8
45	1.85×10^{9}	81.1	0.93×10^{8}	65.0
Original*	2.28×10^{9}	100.0	1.43×10^{8}	100.0

* Specimen S5 before sucrose treatment and diluted with virus diluent instead of STE or sucrose solutions.

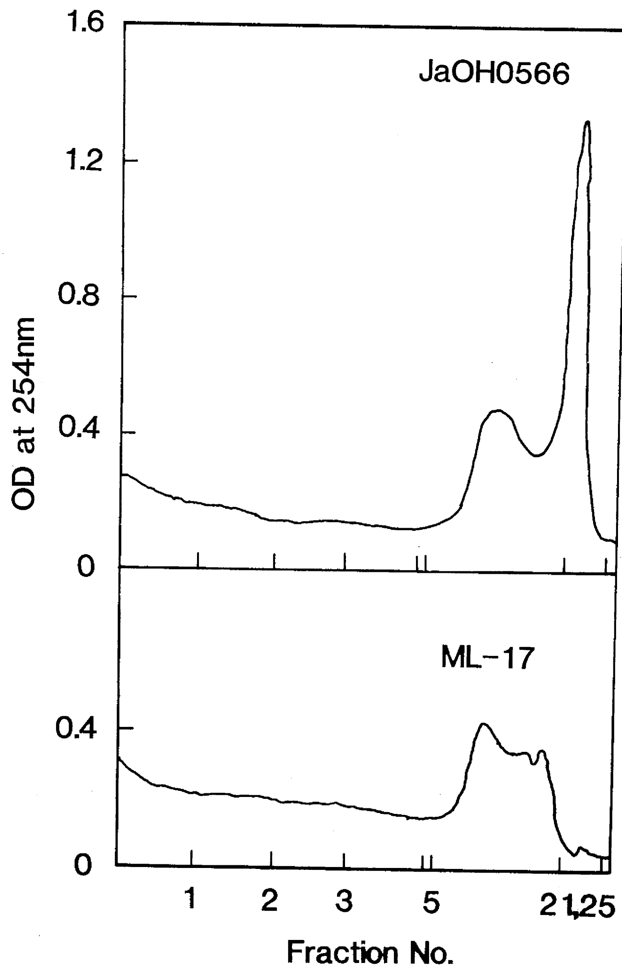

Fig. 1. Sucrose density gradient of JaOH0566 and ML−17 strains of Japanese encephalitis virus. Specimen S5 of the JaOH0566 (upper panel) or the ML−17 (lower panel) strain was overlayed on 10 ml of 30−45% linear sucrose gradient and 2 ml of 15% sucrose column and centrifuged at 20,000 rpm for 20 hr at 4℃ with SW28 rotor of a Beckman L8−80M ultracentrifuge and fractions were collected by an ISCO density gradient fractionator model 640 and OD_{254} was recorded. Volume of the fractions 1 to 4 was 6.0 ml and from 5 to 25 was 0.6 ml. OD peak around fraction 21−23 corresponds to the complete virion.

Discussion

In order to investigate the molecular basis of attenuation of the ML−17 strain, repeated attempts have been made in our laboratory to concentrate and purify this strain by PEG precipitation without successful recovery of the purified virion in quantity. Therefore, we tried to see the effect of PEG on the ML−17 strain as well as on its parental JaOH0566 strain and found that the ML−17 was recovered much less than the JaOH0566 strain. From these data, together with the profile of sucrose density gradient sedimentation and the stability of the viral infectivity in sucrose solutions, we assume that

the virion of ML−17 strain was less stable than that of JaOH0566 strain, specially by PEG treatment. The results suggest that these 2 strains could have some differences in their virion structural proteins, which appears to be supported by the nucleotide sequence analysis on these 2 strains (Hori *et al.,* 1988). Several investigators applied PEG precipitation for virus concentration, for example, plant viruses (Leberman *et al.,* 1966), vesicular stomatitis virus (McSharry and Benzinger 1970), and bacteriophage (Yamamoto and Alberts 1970). In the previous experiment of virus growth and stability of several strains of JE virus in virus diluent, the ML−17 and the JaOH0566 strains did not show any significant difference (Shameen *et al.,* 1988). Therefore, reduced stability of the ML−17 became apparent after it was treated with PEG. Hahn *et al.,* (1987) compared nucleotide sequences of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. They argued that at least some of the difference in virulence between the two strains of a virus results from changes in the envelope protein that affect virus binding to host receptors and such differences in receptor binding could result in the reduced neurotropism and vicerotropism exhibited by the vaccine strain. Our study is especially interesting because it represents some *in vitro* phenotypic difference between an attenuated and its parental JE virus strains, which may be due to the mutation either in the structural or nonstructural gene after passages in the tissue culture.

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