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Comparison of Japanese Encephalitis Virus Isolates from Japan and Thailand by Peptide Mapping

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Abstract: Purified preparations of Japanese encephalitis (JE) virus isolates from Japan and Thailand were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to reveal their structural proteins. Three structural polypeptides, V1, V2 and V3, were observed with all the strains under reducing condition. Limited digestion of V3 by *Staphylococcus aureus* V8 protease showed that Nakayama strain was more easily cleaved than other isolates. At higher concentration of the protease, V3 structural protein of all the strains were cleaved with similar peptide pattern.

Key Words: Japanese encephalitis virus, Protease, Structural proteins, Strain difference.

INTRODUCTION

Japanese encephalitis (JE) virus is a member of Flaviviridae with single-stranded RNA genome of 42S, and was also classified as mosquito-borne group B arbovirus (Friedman, 1968; Shapiro *et al.*, 1971). Since this virus was first isolated in 1935, a lot of strains have been isolated from mosquitoes, swine, horses, as well as fatal human brains in Japan. The virus is known to exist not only in Japan but also in Korea, China, Taiwan, South-east to South Asia (Mitamura *et al.*, 1938; Hammon *et al.*, 1949; Buescher *et al.*, 1959; Igarashi, 1980; Igarashi *et al.*, 1981a, b, c, d). Although many investigations have been carried out from epidemiological to molecular studies, the knowledge on the molecular structure of JE virus and related flaviviruses is rather limited compared with alphaviruses of Togaviridae and other members of mosquito-borne arboviruses. Studies on the polypeptides of flaviviruses such as dengue (Stollar, 1969), St. Louis encephalitis (SLE) (Trent and Quereshi, 1971), Kitano *et al.*, (1974) viruses, have shown three structural polypeptides of V1 (M), V2 (C), and V3 (E), corresponding to membrane,

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core and envelope glycoprotein, respectively. The structural proteins of JE virus have not well been characterised with reference to strain difference, except on the classical (Nakayama) and current (JaGAr-01) strains (Takegami *et al.*, 1982). This paper describes comparative studies on the structural proteins of JE virus strains from Japan and Thailand in order to see the differences as revealed by SDS-PAGE and peptide-mapping by V8 protease.

MATERIALS AND METHODS

Cells: *Aedes albopictus*, clone C6/36, cells were grown at 28°C with cell growth medium of Eagle's minimal essential medium supplemented with 0.2 mM each of nonessential amino acids (Eagle, 1959) and 10% heat-inactivated fetal calf serum (Igarashi, 1978).

Virus: Strains of JE virus were Nakayama Yoken at high passage in suckling mouse brain (SMB), JaOArS982, ThCMP1982 and ThCMAr18084. Their origins were described by Hori and his colleagues (Hori *et al.*, 1985). Nakayama strain grown in SMB and its formalin-inactivated vaccine were also used as references. The seed virus was prepared for each strain by growing in C6/36 to high titer exceeding 10^8 PFU/ml.

Purification of the virus: Confluent cultures of C6/36 cells in Roux bottles were inoculated with seed virus (2 ml/bottle). After two hours adsorption, the cells were covered by 40 ml/bottle of the maintenance medium (the cell growth medium from which serum concentration was reduced to 2%) and incubated at 28°C. Infected culture fluid was harvested at 48 hours after the infection using cytopathic effect as indicator. The fluid (250 to 500 ml volume) was centrifuged at 2,500 rpm for 15 minutes and supernatant was added with polyethylene glycol 6000 and NaCl to 6% and 0.5 M, respectively. The mixture was centrifuged at 10,000 $\times g$ for 30 min, supernatant was removed and the precipitate was dissolved in 6 ml of STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) and centrifuged at 2,500 rpm for 15 min. The supernatant was layered on top of 2 ml of 15% (w/w) sucrose column and 4 ml of 30-50% (w/w) linear sucrose gradient in STE buffer and centrifuged at 25,000 rpm for 12 hours at 4°C in an SW41 rotor of a Beckman L5-50 ultracentrifuge. Fractions of 0.4 ml volume was collected by ISCO density gradient fractionator, model 640, and the peak fractions of OD_{254} were used as purified virus specimen.

SDS-PAGE: Slab gel method (Studier, 1973) with discontinuous buffer system (Laemmli, 1970) was used with 10% gel (acrylamide bisacylamide ratio of 30: 0.8) in 1 mm thickness. Specimens were solubilized under reducing condition (2% SDS and 5% 2-mercaptoethanol) in 0.5 M Tris-HCl, pH 6.8, by heating at 100°C for 1 min. After electrophoresis protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R250 in 10% acetic acid and 30% methanol by diffusion.

V8-protease digestion and peptide mapping: The procedure was described by Cleve-

land *et al.* (1977). Protein band in 1 mm gel was cut and digested with *Staphylococcus aureus* V8 protease in the slot of 1.5 mm gel in the presence of 0.1% SDS followed by electrophoresis and staining.

Chemicals: Acrylamide, bisacrylamide and 2−mercaptoethanol were the products of Wako Pure Chemicals Co. V8−protease was obtained from Sigma Chemicals, USA.

RESULTS

Structural proteins of JE virus strains: The Thai isolates (ThCMP1982, ThCMAr-18084) and Japanese strains (Nakayama, JaOArS982), grown in C6/36 cells and Nakayama passaged in SMB were analysed for their structural proteins by SDS−PAGE under reducing condition with 2−mercaptoethanol. Three bands of structural proteins were revealed for all the strains as shown in Fig. 1, with estimated molecular weight of 8K, 18K, and 54K for V1, V2 and V3, respectively. Several additional bands with molecular

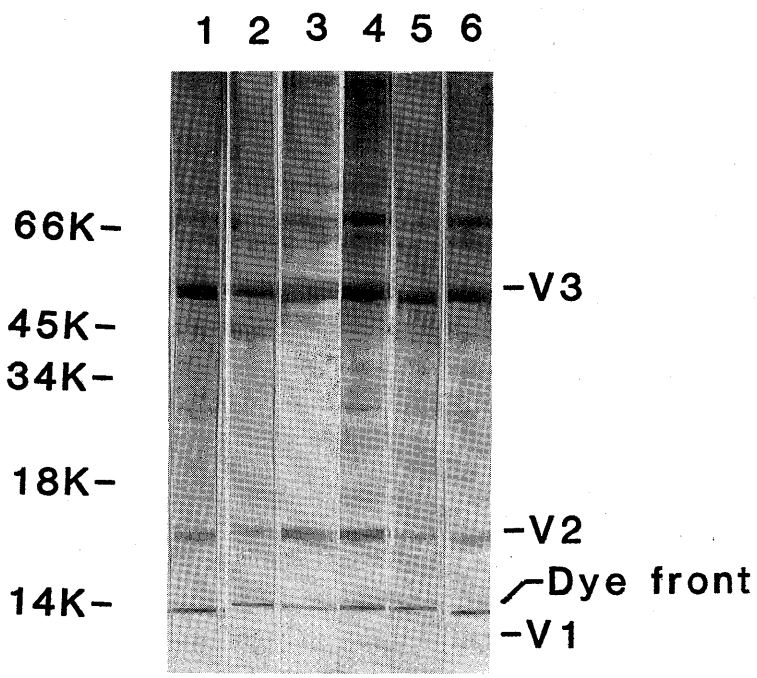

Fig. 1. Separation of structural proteins of Japanese encephalitis virus strains by SDS-PGAE under reducing condition. Purified preparations of JE virus strains were analysed on 10% SDS-PAGE as documented in the Materials and Method. Specimenes were: lane 1, Nakayama Yoken grown in C6/36; lane 2, Nakayama grown in suckling mouse brain; lane 3, formalin-inactivated Nakayama vaccine; lane 4, JaOArS982 grown in C6/36; lane 5, ThCMP1982 grown in C6/36 and lane 6, ThCMAr18084 grown in C6/36.

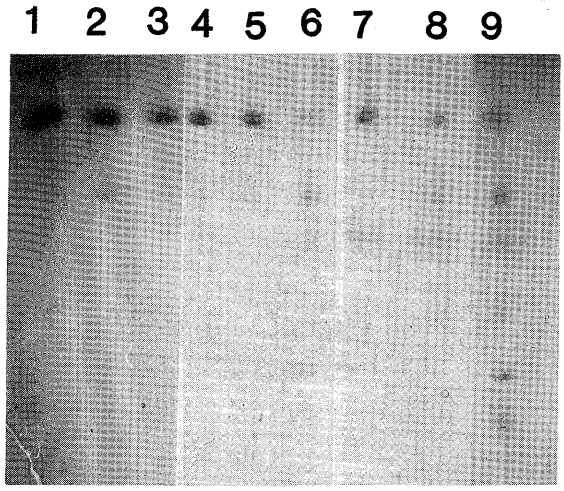

Fig. 2. Peptide mapping of structural protein V 3 of Japanese encephalitis virus strains by limited digestion with *Staphylococcus aureus* V 8 protease followed by SDS-PAGE. Structural protein V 3 of JaOArS982 (lanes 1,4,7), ThCMP1982. (lanes 2,5,8), Nakayama (lanes 3,6,9) were cut out and treated with V 8 protease at room temperature for 30 min in the presence of 0.1% SDS in the stacking gel of 1.5 mm thickness. The final concentration of *Staphylococcus aureus* V 8 protease loaded was: lanes 1 to 3, 0 μg/ml; lanes 4 to 6, 10μg/ml and lanes 7 to 9, 25μg/ml.

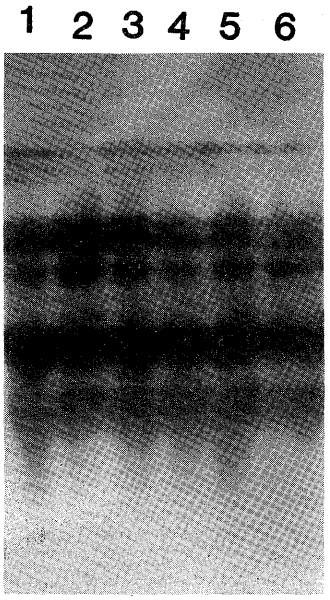

Fig. 3. Peptide mapping of structural protein V 3 of Japanese encephalitis strains by digestion with high concentration of *Staphylococcus aureus* V 8 protease followed by SDS-PAGE. Structural protein V 3 was cut out and treated with 500μg/ml of V 8 protease at 37°C for 90 min in the presence of 0.1% SDS in the stacking gel of 1.5mm thickness. ThCMAr18084, lane 1; ThCMP1982, lane 2; JaOArS982, lane 3; Nakayama Vaccine lane 4; Nakayama SMB, lane 5 and Nakayama C6/36, lane 6.

weights of 74K, 70K, 38K, and 30K would probably represent contaminants of cellular protein.

Peptide mapping by V8—protease digestion: Protein band corresponding to V3 structural protein was cut out and digested by V8—protease at various concentrations (25 μg/ml and 10 μg/ml or 0 μg/ml) and results were shown in Fig.2. At low concentrations of the protease, only Nakayama strain showed cleaved pattern. However, higher concentration of the protease (500 μg/ml) cleaved V3 polypeptide of all the 6 specimenes and no significant difference was observed between the strains of JE virus tested, (Fig.3.).

DISCUSSION

Hori *et al.* (1985) showed that JE virus isolates in Japan were significantly different from isolates in Thailand by RNA oligonucleotide fingerprint analysis. However, present result did not show any significant difference between 4 representative strains from Japan and Thailand. Therefore, peptide analysis is less sensitive than RNA fingerprint to reveal strain difference, or the difference in the RNA fingerprint could mostly represent the difference in nonstructural regions of viral genome, although previous studies on Getah virus structural protein revealed the difference between a Malaysian isolate and Japanese strains by peptide—mapping analysis (Srivastava and Igarashi, 1985). Susceptibility of V3 polypeptide of Nakayama strain could be due to its mouse passage level, because other three strains were all wild isolates which have not been passed more than several generations. Possible effect of mouse passage on the susceptibility of V3 polypeptide of fresh isolates should be examined in the future.

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日本とタイ国で分離された日本脳炎ウイルス株間のペプチドマッピングによる比較

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日本とタイ国で分離された日本脳炎ウイルス株を SDS ポリアクリルアミドゲル電気泳動により分析した，検査したすべての株において三種の構造タンパク V1, V2, V3 を認めた．黄色ブドウ球菌 V8 タンパク分解酵素によって中山株の V3 タンパクは他の株のものよりも容易に加水分解された．より高濃度の V8 タンパク分解酵素を用いるとすべての株の V3 タンパクは加水分解されて類似のペプチドパターンを示した．

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