IDENTIFICATION OF A FLAVIVIRUS ISOLATED FROM MOSQUITOS IN CHIANG MAI THAILAND

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Abstract. A virus isolate, ThCAr105/92, from a pool of mosquitos, *Culex tritaeniorhynchus*, collected in Chiang Mai, Thailand in 1992, appeared to be a member of the genus *Flavivirus* of the family Flaviviridae, based on the reverse transcription polymerase chain reaction (RT-PCR) using flavivirus cross-reacting primer pairs, electron microscopic examination, and serological tests. However, RT-PCR using Japanese encephalitis (JE) virus-specific primers showed that the isolate was different from JE virus. Sucrose density gradient sedimentation of the virus replicated in C6/36 cells indicated that the virus is relatively unstable in the infected culture fluids at 37°C. Antibody prepared against this virus and a virus seed for the isolate were tested by cross neutralization against a panel of flaviviruses and the results showed that the new isolate was a distinct subtype of Tembusu virus.

INTRODUCTION

Diseases caused by flavivirus infections are among the serious health problems in the world. The genus *Flavivirus* of the family Flaviviridae is composed of more than 70 viruses, and several of these are pathogenic to humans (Murphy *et al,* 1995). Multiple flavivirus infections are prevalent in many parts of Asia (Innis *et al,* 1989). Dengue fever (DF)/ dengue hemorrhagic fever (DHF) and Japanese encephalitis (JE) affect a large number of people resulting in substantial morbidity and mortality. Dengue and its severe and sometimes fatal forms, DHF and dengue shock syndrome (DSS), alone affect nearly 80 million people over a year (Monath *et al,* 1994). Because of high fatality and grave sequelae, JE epidemics are considered serious health problems in several developing countries in Asia. Many encephalitis cases are reported every year in northern Thailand since the first outbreaks in 1969 (Yamada *et al,* 1971, Grossman *et al,* 1973). JE and dengue viruses are found to co-exist in these areas and the outbreaks caused by these viruses have taken place simultaneously during the rainy seasons in Thailand (Fukunaga *et al,* 1984).

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ThCAr105/92 was an unidentified flavivirus isolated from a pool of *Culex tritaeniorhynchus*, collected during an entomological and virological study of JE in Chiang Mai, Thailand in August 1992. The reverse transcription polymerase chain reaction (RT-PCR) using flavivirus cross-reacting primers indicated that it was a flavivirus. However, inability to amplify its genome by RT-PCR using JE-specific primers indicated it was different from existing strains of JE virus (Ali *et al,* 1995). Intra cerebral inoculation of this virus into Balb/c mice resulted in the development of neurological symptoms followed by a fatal outcome three to four days after inoculation, suggesting that the virus is pathogenic and neuroinvasive.

The objective of this study was to characterize the virus, ThCAr105/92. In this paper we report its isolation, biological characterization and identification.

MATERIALS AND METHODS

Cell culture

Aedes albopictus clone C6/36 cells (Igarashi, 1978) were grown at 28°C in Eagle's minimum essential medium (MEM) supplemented with 0.2 mM each of nonessential amino acids and 10% heat-inactivated fetal calf serum (FCS) in rubber stoppered 16x20 ml tubes. BHK-21 cells were grown at 37°C in the same medium. After inoculation, cells

were further maintained in the medium supplemented with 2% FCS (maintenance medium).

Virus isolation

Pools of unengorged *Cx. tritaeniorhynchus* were processed for virus isolation. The presence of the flavivirus antigen in the infected culture supernatant was screened by microsandwich ELISA (Voller *et al*, 1976). Any specimen showing more than a two fold ELISA-OD above that of the negative control was further passaged in C6/36 cells to confirm the production of flavivirus antigen in the culture supernatant. The cultures which showed consistent production of flavivirus antigen were inoculated into bottle cultures of C6/36 cells. The infected culture fluid was collected after 7 days incubation at 28°C and kept at -70°C as seed virus.

Sample preparation for viral growth experiment

Cell cultures were prepared in 12 well plates (Falcon, USA) and 100 µl seed virus of ThCAr105/92 was inoculated into each well. After 2 hours adsorption, cells were covered with 1.5 ml of the maintenance medium and incubated at 28°C or 37°C. Infected culture fluid and cells were collected every day for a period of 7 days. Sample were stored at 4°C for viral antigen assay and at -80°C for virus infectivity assay.

Assay of viral hemagglutination (HA)

Hemagglutinating activity of the virus was assayed according to the method of Clarke and Casals (1958) using goose red blood cells and 96 well microtiter plates. The optimum pH for HA of the new isolate was found to be 6.4.

Infectivity assay by focus forming unit (FFU)

Infectivity of the isolates was measured by focus forming test as modified from the procedure of Okuno *et al* (1985). The overlay medium containing 0.5% methyl cellulose 4,000 in the maintenance medium (2% FCS-MEM) was used after the infection with 50 µl/well of diluted virus specimens. Infectivity titer was expressed as focus forming units (FFU/ml).

Sucrose gradient sedimentation analysis

Gradient columns (volume 4.4ml) of 30-50% sucrose in STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) were prepared, overlayed with 0.6 ml of sample and centrifuged at 40,000 rpm for 3.5 hours at 4°C in SW 50.1 rotor of a Beckman model L8 ultracentrifuge. Fractions were collected and examined by HA.

Mouse immunization

Seed virus of ThCAr105/92 was inoculated intracranially into suckling Balb/c mice (0.02ml/brain), and they were observed for the neurological symptoms. Brains were harvested from sick mice, and a 10% homogenate was prepared in PBS for use as immunogen. The infected mouse brain homogenate was emulsified in Freund's complete adjuvant and 200µl was inoculated intraperitoneally into Balb/c mice for a total of 5 injections at 1 week intervals. The mice were bled 1 week after the last injection and sera were separated and pooled for serological assay.

Electron microscopic examination

Infected C6/36 cells were fixed in 1.5 % glutaraldehyde containing 0.01% $CaCl_2$ in cacodylate buffer (0.1M sodium cacodylate, pH 7.2). Three washings were done with cacodylate buffer by centrifuging at 450*g* for 15 minutes each time. Post fixation was done in 1% osmium tetroxide in cacodylate buffer for 45 minutes at room temperature.

The fixed specimens were dehydrated through graded ethanol solutions and finally in absolute acetone. Then they were infiltrated and embedded in epoxy resin. Ultrathin sections were prepared using a Reichert Ultra cut E ultramicrotome, followed by the staining with uranyl acetate and lead citrate. The specimens were observed and photographed under a JEM 100 CX electron microscope (JEOL Ltd, Tokyo) at an acceleration voltage of 80 kV.

Effect of 5-iodo-2′-deoxyuridine (IUdR) and actinomycin D (AMD) on the production of viral HA in the infected C6/36 cells, and ether-sensitivity test of viral HA

C6/36 cell cultures were prepared in 6 well plates and incubated at 28°C. When the cells became confluent, growth medium was removed and 100 µl of seed virus was inoculated. After 2 hours adsorption, cells were covered by 2 ml of the maintenance medium/ well. Stock solutions of $10^{-2.5}$M IUdR and 10 µg/ml AMD were used. IUdR was used in the working solution at $10^{-4.5}$M and $10^{-3.5}$M, whereas actinomycin D was used at three different working concentrations of 0.01, 0.1 and 1µg/ml. Preliminary experiment showed that the HA titer produced in the infected C6/36 cell culture fluid was maximum 2 days after infection at 28°C. Therefore, the HA titer produced 2 days after infection in the culture fluid was examined for testing the effect of IUdR and AMD. The sensitivity of the virus was examined by emulsifying stock virus with equal volume

of ether before testing the residual virus HA and infectivity in the aqueous phase.

Identification of the isolate

The virus specimen and its mouse antiserum were sent to the Division of Vector-Borne Infectious Diseases, Centers for Diseases Control and Prevention (CDC), Fort Collins, CO, USA. The isolate was screened in typing neutralization tests using antibodies to various flaviviruses (homologous titers for these antibodies were predetermined), after which the viral isolate and its antibody were tested by cross neutralization tests using Tembusu virus and antibody.

RESULTS

Sucrose gradient centrifugation

Infected culture fluid was collected 2 days after post infection from C6/36 cells incubated at 28°C and 37°C. The samples were fractionated by sucrose gradient sedimentation and each fraction was assayed for HA. The results showed that sedimentation rate of viral HA produced at 37°C in C6/36 cells was less than that of HA produced at 28°C, indicating degradation of the virus at higher temperature.

Effect of 5-iodo-2′-deoxyuridine (IUdR) and actinomycin D (AMD) on the virus HA production in C6/36 cells

The results of HA titration on C6/36 cell culture fluid collected after 2 days post infection showed that IUdR did not significantly inhibit the production of viral HA at concentrations of $10^{-3.5}$M or $10^{-4.5}$M. Two different concentrations of AMD (0.01 to 0.1μg/ml) slightly enhanced HA production, whereas the highest concentration of AMD (1μg/ml) slightly inhibited HA production.

Electron microscopic examination

C6/36 cells infected with ThCAr105/92 were incubated for 48 hours and observed under the electron microscope. Numerous spherical viral particles measuring approximately 50 nm in diameter were observed in clusters and free in the cytoplasm (Fig 1).

Identification

Initially, the isolate ThCAr105/92 was screened by the neutralization test using antibodies to various flaviviruses. The viral isolate was significantly neutralized by flaviviral antibody to Tembusu virus (Table 1). Cross-neutralization testing of the virus isolate, ThCAr105/92, to prototype Tembusu virus showed that the antibody against Tembusu virus neutralized ThCAr105/92 and Tembusu virus prototype equally (Table 2). However, antibody against ThCAr105/92 significantly neutralized ThCAr105/92 to a greater extent than it did prototype Tembusu virus. Thus, the new flaviviral isolate ThCAr105/92 was identified as a distinct subtype of Tembusu virus.

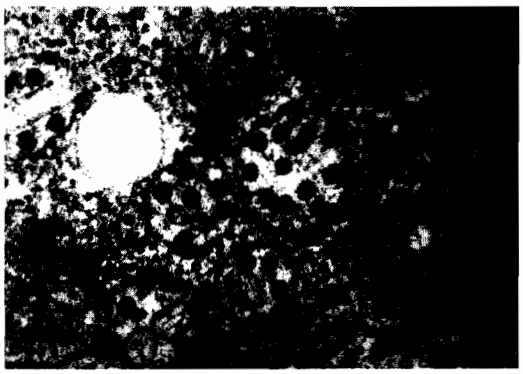

Fig 1–Electron microscopic observation of C6/36 cells 2 days after infection with the virus isolate, ThCAr105/92.

DISCUSSION

The genus *Flavivirus* of the family Flaviviridae comprises over 70 viruses and several of them, such as the dengue viruses, Japanese encephalitis virus, St Lious encephalitis virus and yellow fever virus, are important pathogens (Murphy *et al*, 1995). Recently Kuno *et al* (1998) reported revision on the phylogeny of the genes *Flavivirus* on the basis of their mode of transmission and geographical distribution. Tembusu virus belongs to the family Flaviviridae and genus *Flavivirus*. ThCAr105/92 is 90% homologous with Tembusu virus prototype on the basis of nucleotide sequencing (Kuno, unpublished data).

A study carried out by Leake *et al* (1986) reported that 18 isolates of Tembusu virus were obtained from Kamphaeng Phet Province, Northern Thailand, along with other flaviviruses, during an epidemiological study in 1982. These viruses were isolated over a ten days period and the last isolate was obtained one week before the peak admission of human encephalitis case in Kamphaeng Phet Provincial Hospital. However, no further study has been

Table 1

Screening of the virus isolate, ThCAr105/92, by the neutralization test against antibodies to various flaviviruses from the same geographical region where the isolate was recovered

Flavivirus antibodies	90% PRNT titer	
	heterologous	homologous
Alfuy	-	5,120
Apoi	-	5,120
Gadgets Gully	-	160
Dengue 1	<4	80
Dengue 2	-	640
Dengue 3	4	20
Dengue 4	-	2,560
Edge Hill	<4	80
Israel turkey meningoencephalitis	40	1,280
Japanese encephalitis	<10	160
Jugra	8	40
Karshi	10	>320
Kokobera	-	1,280
Kunjin	40	5,120
Murry Valley encephalitis	-	2,560
Negeshi	-	640
Phnom Penh bat	-	640
Royal Farm	<10	320
Sumeraz Reef	<10	320
Sepik	-	640
Sokoluk	-	5,120
Startford	-	640
Tembusu	≤640	640
West Nile	-	5,120
Yokose	20	2,560
ThCAr105/92 (homologous)	10,240	10,240

PRNT: Plaque-reduction neutralization test
Flavivirus homologous neutralization titers were predetermined
- = <20.
PFU of ThCAr105/92 in test = 72

Table 2

Cross neutralization relationship of the new isolate, ThCAr105/92, to prototype Tembusu virus.

Virus	PFU in tests*	90% PRNT titer of antibody	
		TMU	ThCAr 105/92
TMU	113,72	640	450
ThCAr105/92	60,82	640	2,560

*PFU = counts given for each of two different neutralization tests.
PRNT = plaque reduction neutralization tests.
TMU = prototype Tembusu virus, strain MM1755.
450 geometric mean titer of three of three determinations.

done to determine whether these viruses are pathogenic to human beings.

The result presented here indicate the presence of a new subtype of Tembusu virus in Chiang Mai, Thailand. It would be interesting to test human sera or cerebrospinal fluid from encephalitis cases, who did not possess anti-JE IgM antibodies, for the presence of anti-Tembusu IgM antibodies in order to determine the possible role of this virus as a cause of human encephalitis.

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