PRODUCTION OF VIRAL ANTIGENS IN CULTURE FLUID OF C6/36 MOSQUITO CELL LINE INFECTED WITH DENGUE TYPE 4 VIRUS STRAINS ISOLATED FROM PATIENTS WITH DIFFERENT CLINICAL SEVERITIES

C. CHANYASANHA[1*], F. HASEBE[2], R. MATIAS[3,4], A. IGARASHI[2]

[1]Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand; [2]Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan; [3]Institute of Biology, College of Science, University of the Philippines, Manila, Republic of the Philippines; [4]Department of Molecular Epidemiology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

Received December 1, 1998; accepted April 12, 1999

Summary. – Viral antigen production was examined in the culture fluid of *Aedes albopictus* clone C6/36 cell line incubated at 28°C and 37°C after infection with four strains of dengue type 4 (DEN-4) virus which were isolated from patients with different clinical severities. During the observation period from day 1 to day 18, the number of infected cells at each day for all four strains did not show any significant difference ($P > 0.05$). Antigen production as determined by the hemagglutination (HA) test and sandwich enzyme linked immunosorbent assay (ELISA) was higher at 28°C than at 37°C for all four DEN-4 virus strains examined. The amount of viral antigen produced was highest for CT93-74 strain (dengue hemorrhagic fever syndrome (DHF) grade II) and was significantly different in comparison to other strains ($P < 0.001$). This strain was followed by CT93-158 and CT93-129 strains (both of DHF grade I), and CT93-77 strain (dengue fever (DF)). The viral antigen production was apparently proportional to the clinical severity of the patient from whom the virus was isolated. These results show that CT93-74 strain could be used to produce DEN-4 virus antigen of sufficiently high titer in the C6/36 cell culture instead of classical extraction of this antigen from suckling mouse brains.

Key words: dengue type 4 virus; antigen; cell culture; incubation temperature; HA test; sandwich ELISA

Introduction

Dengue virus infection has become one of the serious infectious diseases and public health problems because of increasing number of the patients, enlarging epidemic areas, and appearance of severe clinical manifestations, such as DHF/dengue shock syndrome (DSS) (Halstead, 1966, 1980, 1992, 1993; World Health Organization, 1966). Laboratory confirmation of dengue virus infection has been carried out by virus isolation and serology. Although time, expertise, and facilities required for the virus isolation were greatly simplified by viral genome detection using reverse transcriptase-polymerase chain reaction (RT-PCR), the serology has still most frequently been used for laboratory diagnosis of dengue. The classical serology by the hemagglutination-inhibition (HAI) test has recently been replaced or supplemented by the IgM capture ELISA, because of its diagnostic potential with single serum specimens (Rojanasuphot *et al.*, 1981; Chanyasanha *et al.*, 1984a,b; Ahandrik *et al.*,1987; Shope and Sather, 1979; Bundo and Igarashi, 1985; Tanaka, 1993; Chanyasanha, 1997).

The antigens used in these serological tests were originally prepared and extracted from infected suckling

*E-mail: phccy@mahidol.ac.th; fax: +662-245-8351.

Abbreviations: BABS = bovine albumin borate saline; DEN-4 = dengue type 4; DF = dengue fever; DHF = dengue hemorrhagic fever; DSS = dengue shock syndrome; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; HA = hemagglutination; HAI = hemagglutination inhibition; HRPO = horseradish peroxidase; PBS = phosphate-buffered saline; RT-PCR = reverse transcription-polymerase chain reaction

mouse brains, which requires animal handling and large quantities of flammable organic solvents. Several reports indicated that DEN-1 virus and other arbovirus antigens could be produced in *A. albopictus* clone C6/36 cell line of mosquito origin to sufficient titers for use in laboratory diagnosis or serological surveillance (Thien *et al.*, 1979; Della-Porta and Westaway, 1972). Another series of study showed that production of DEN-3 and DEN-4 virus antigens in the infected C6/36 cell line was enhanced by elevating the temperature of incubation of the infected cells (Corner and Mah, 1987; Mohamed *et al.*, 1995). This phenomenon was explained by increased viral RNA synthesis in the infected cells (Mangada *et al.*, 1995). A study of antigen production with seven DEN-4 strains in C6/36 cell line at 28°C and in Vero cell line at 37°C showed that viral antigen production was different from strain to strain, but it was still of insufficiently low titer for use in routine serology (Kyaw-Zin-Thant *et al.*, 1995)

The objectives of this study were to find DEN-4 strains producing virus antigen of sufficiently high titer and optimal temperature for the virus antigen production in C6/36 cell line. The cells were incubated at 28°C and 37°C after infection with four different DEN-4 virus strains, respectively, which were isolated from patients with different clinical severities.

Materials and Methods

Virus strains. Four strains of DEN-4 virus used in this study were isolated from sera of dengue patients admitted in 1993 to the Children's Hospital, the Queen Sirikit Institute, Bangkok, Thailand, and showing different clinical manifestation (Chanyasanha *et al.*, 1995). The CT93-77 strain was isolated from a DF, while the strains CT93-129 and CT93-158 were from DHF grade I cases, and the CT93-74 strain was from DHF grade II case, respectively. The presence of viral genome in each virus strain was confirmed by RT-PCR (Chomczynski, 1993; Morita *et al.*, 1991). Virus materials were and kept at -80°C until use. Before the study, the same dose of each strain was used for infection of *A. albopictus* clone C6/36 cells in 60-mm Petri dishes for a week at 28°C in 5% CO_2 incubator. As maintenance medium we used a cell growth medium in which heat-inactivated fetal calf serum (FCS) concentration was reduced to 2%. The infected culture fluid was harvested and used as the seed virus in the experiments.

Cell culture. The *A. albopictus* clone C6/36 cell line was grown at 28°C in Eagle's Medium with Earle's salts supplemented with 0.2 mmol/l nonessential amino acids and 9% of heat-inactivated FCS (Igarashi, 1987). For production of the seed virus, the cells were grown in Petri dishes as described before, while for virus growth experiments, the cells were grown in 24-well plates (Falcon) and incubated in a 5% CO_2 incubator. The cell density before the virus inoculation was approximately 10 x 10^5 cells per well.

Virus growth experiment. The growth medium was removed from C6/36 cell cultures in 24-well plates by aspiration, and 0.1 ml of each seed virus was inoculated in each well (with 18 replicate wells in 2 plates). Mock-infected control cells were inoculated with the same volume of the maintenance medium. The virus adsorption was carried out for 2 hrs at 28°C with the spreading of the inoculum over the cell sheet every 30 mins. Then the cells were covered by 1 ml per well of the maintenance medium with 2% of heat-inactivated FCS. Of two replicate plates one was incubated at 28°C while another at 37°C, both in a 5% CO_2 incubator.

Specimens collection. The maintenance medium was not changed during the whole experiment. It was collected every day for 18 consecutive days, from day 1 to day 18 post infection (p.i.). The collected medium samples were used for assay of viral antigen by a sandwich ELISA and for assay of HA titer by HA test (Clarke and Casal, 1958) as described below. The remaining cells in each well were resuspended by pipetting and their number was determined by counting in a hemocytometer.

Sandwich ELISA. A microsandwich ELISA of DEN viral antigen was used in this study (Voller *et al.*, 1976) with a slight modification consisting of incubation at room temperature for 1 hr and reagent volume of 100 µl/well. Washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) was done 3 times for 3 mins each. Briefly, a 96 well flat-bottom microplate (Nunc) was sensitized overnight by antiflavivirus IgG (20 µg/ml) in 0.05 mol/l carbonate-bicarbonate buffer pH 9.6 containing 0.01% NaN_3. This antiflavivirus IgG was purified from high-titer DHF patients sera by chromatography on DEAE Sephacel column (Pharmacia). The plate was inactivated by Blockace (Yukijirushi, Japan), followed by washing. Test specimens, negative control (uninfected culture fluid) and positive control (standard DEN-2 viral antigen) diluted 2-fold in the maintenance medium were distributed in wells and incubated. After washing, a horseradish peroxidase (HRPO) (Type VI, Sigma)-conjugated antiflavivirus IgG at 1:1000 dilution in PBS-T was added. This reagent was prepared by the method of Wilson and Nagane (Wilson and Nagane, 1978). After incubation and washing, the substrate solution of 0.5 mg/ml *o*-phenylene diamine dihydrochloride (Sigma) and 0.02% H_2O_2 in 0.05 mol/l citrate-phosphate buffer pH 5, was added. After 1-hr incubation in the dark at room temperature, the reaction was stopped by 1N H_2SO_4. A_{492} and A_{630} values of each well were measured in an ELISA reader. Antigen titers of specimens were read from a calibration curve obtained with a standard DEN-2 viral antigen and were expressed as reciprocals of the highest positive dilutions.

HA test. Medium samples were diluted 2-fold with 0.04% bovine albumin borate saline (BABS) and were titrated by HA test in microtiter plates (Clarke and Casals, 1958). HA titers were expressed as reciprocals of highest positive dilutions.

Statistical analysis. The analysis of variance (ANOVA) was used as statistical test for differences in ELISA and HA titers and cell numbers of DEN-4 strains. The *t*-test was used for antigen titers and cell numbers of both temperatures.

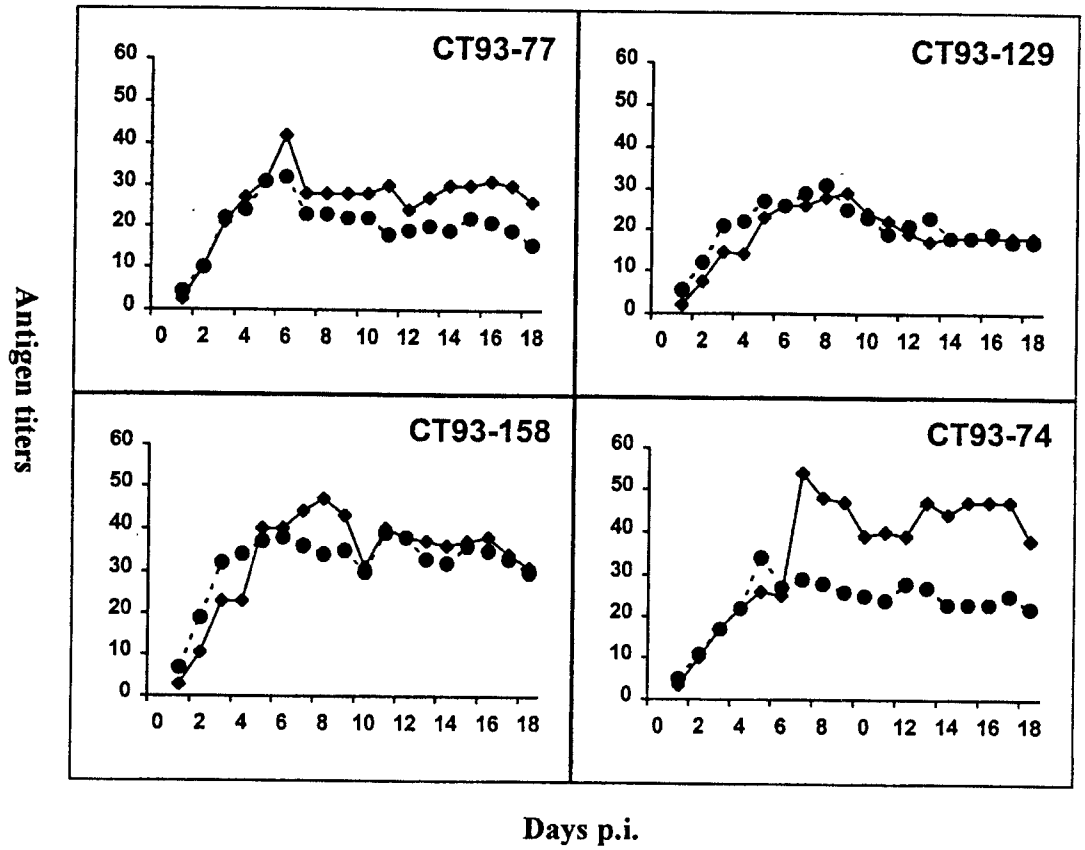

Fig. 1
Antigen production in C6/36 cells infected with various DEN-4 virus strains at 28°C and 37°C as measured by ELISA
28°C (squares) and 37°C (circles).

Results

Cell number in the course of infection with different virus strains at 28°C and 37°C

During the experiment, the number of C6/36 cells in each well was determined by counting daily from day 1 until day 18 (data not shown). Neither the four different virus strains nor the two different inocubation temperatures caused any significant difference in the cell number.

Virus antigen production by different virus strains measured by ELISA

Fig. 1 shows the level of DEN-4 virus antigen production by C6/36 cells incubated at 28°C and 37°C. Until day 6 p.i. all the 4 different strains produced similar antigen titers at 28°C and 37°C. However, after day 6, CT93-74 strain produced significantly higher titers than other strains at both temperatures ($P < 0.0001$). The level of antigen production as measured by ELISA titer was highest for CT93-74 (titer 54) at day 7, followed by strains CT93-158 at day 8 (titer 48) and CT93-77 at day 6 (titer 42); it was lowest for CT93-129 at day 8 (titer 28). The viral antigen was detectable from day 1 until day 18 p.i. (the end of the experiment); it reached a peak at days 6–8.

HA antigen production by different DEN-4 virus strains

All four strains of DEN-4 virus produced a significant HA titer at 28°C and the optimal pH was 6.4. In contrast, the HA antigen production was almost undetectable when the infected C6/36 cells were incubated at 37°C. ($P > 0.05$). With the incubation temperature of 28°C, the HA antigen became detectable from days 2–4 in dependence on the virus strain used and remained detectable up tp day 12 except CT93-74 strain which produced detectable HA antigen up to day 16. CT93-74 strain showed the highest titer (64 HAU/0.05 ml) at days 8–9. The highest titers of the other strains were lower: 32 HAU/0.05 ml for CT93-158 strain at days 8–9, and 8

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