Elevated Incubation Temperature Enhanced Antigen Production of Dengue Type 2 and 3 Viruses in the Infected *Aedes albopictus* Clone C6/36 Cell Cultures

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Abstract: By elevating the incubation temperature of infected *Aedes albopictus* clone C6/36 cells to 32℃ or 37℃, amount of dengue type 2 and type 3 viral antigens produced in the culture fluid was increased. While the maximum titer of type 1 dengue virus antigen was not significantly elevated although they reached maximum titer 1−2 days earlier. Type 4 dengue viral antigen was almost undetectable under the conditions examined, even though its infectivity was produced in the infected culture fluid.

Key Words: Dengue virus antigens, *Aedes albopictus* clone C6/36 cells, incubation temperature

INTRODUCTION

Dengue virus infection has been a serious health problem in many tropical countries worldwide, by increasing number of cases, enlarging epidemic areas, and appearance of severe manifestation of dengue hemorrhagic fever or DHF (Halstead, 1966; 1980; 1992; World Health Organization, 1966). Laboratory diagnosis on dengue is required to provide accurate information of the disease agents, to the clinicians for proper case management, and to the epidemiologists and health administrators for effective control measures (Igarashi, 1994). Serodiagnosis has most commonly been used for this purpose, either by the haemagglutination inhibition (HI) test (Clarke and Casals, 1958; Shope and Shather, 1979; World Health Organization, 1986), or by IgM-ELISA which has recently been introduced (Burke, 1983; Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989). Most of these tests utilized assay antigens prepared from infected suckling mouse brains, which requires animal raising and extraction procedures with large quantities of acetone or ether. In order to overcome these difficulties, production of dengue antigens in the infected *Aedes albopictus* clone

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C6/36 cell line (Igarashi, 1978) has been tried (Soe Thein *et al.*, 1979). The study showed that dengue type 1 virus antigen was produced in significant titer which can be used in routine diagnosis or epidemiological surveillance. However, similar attempts to produce other serotypes of dengue antigen was not so successful.

In this study, effect of elevating the incubation temperature on the amount of dengue antigen production in the infected C6/36 cell cultures was examined in order to find out better conditions to produce dengue antigen in cell culture system.

Materials and Methods

Dengue virus strains: The following dengue virus strains were used; type 1 (D1) Hawaiian, type 2 (D2) New Guinea B, type 3 (D3) H-87, and type 4 (D4) H-241. All strains were grown in *Ae. albopictus* clone C6/36 cells and the seed virus was stored in aliquots at −70℃.

Cell cultures: *Ae. albopictus* clone C6/36 cell line (Igarashi, 1978) was grown at 28℃ with Eagle's medium in Earle's saline supplemented with 0.2 mM each nonessential amino acids and 9% heat-inactivated fetal calf serum. The cells were grown either in rubber-stoppered bottles in ordinary incubator or in multiwell dishes in 5% CO_2−atmosphere. BHK21 cells were grown at 37℃ using the same medium as C6/36 cells.

Virus inoculation and harvesting specimens: Growth medium was romoved from C6/36 cell cultures and seed virus was inoculated. The inoculum size was 1 ml / 500 ml bottle, 0.2 ml / 2 ounce bottle, or 0.1 ml/well on 24-well plate. Adsorption was carried out for 2 hours at 28℃ spreading the inoculum over cell sheet every 30 min. Then, the cells were covered by the maintenance medium (cell growth medium from which serum concentration was reduced to 2%) using 40 ml / 500 ml bottle, 5 ml / 2 ounce bottle, or 1 ml/well on 24 well plate, respectively. The infected cells were incubated either at 28℃, 32℃ or 37℃ for appropriate periods. The infected fluids were collected, and cell sheets were scraped into phosphate buffered saline (PBS), pH 7.4, using rubber policeman. The specimens were stored at −70℃ for infectivity assay or at 4℃ for antigen assay, respectively.

Assay of haemagglutination activity (HA): Clarke and Casals method (1958) was followed using goose red blood cells. Preliminary test showed that the optimal pH of the HA was 6.4 for D1 and D2, while pH 6.6 was optimal for D3.

Assay of ELISA antigen: Micro sandwich method of Voller *et al.* (1976) was followed using incubation at room temperature for 1 hour and reagent volume of 100 μl/well for each reaction step. A 96-well flat bottom plate (Nunc, Denmark) was sensitized by anti-flavivirus IgG (20 μg/ml) in 0.05M carbonate-bicarbonate buffer, pH 9.6, containing 0.01% NaN_3. The IgG was purified from high titered DHF patient's serum by chromatography on a DEAE Sephacel column (Pharmacia, Sweden). The plate was inactivated by Block Ace (Yukijirushi, Japan), followed by washing with PBS-Tween (0.05% Tween 20 and 0.01% NaN_3 in PBS) using a ICN Titertek Microplate washer (Flow, USA). Test speciments, control positive specimen in serial 2-fold difution steps in the negative control, and negative control of mock-

infected culture fluid were distributed in duplicate wells. After washing as above, the plate was reacted with horseradish peroxidase (HRPO)-conjugated antiflavivirus IgG at 1:100 dilution in PBS-Tween. This reagent was prepared by conjugating the above-mentioned antiflavivirus IgG with HRPO (Sigma, type VI, USA), using Wilson and Nakane's method (1978). After washing as above, peroxidase reaction was carried out by the substrate solution of 0.5 mg/ml *o*-phenylenediamine dihydrochloride and 0.02% H_2O_2 in 0.05M citrate phosphate buffer, pH 5. The OD_{492} on each well was recorded by an ELISA reader using 630 nm as a reference wavelength. The ELISA titer of the test specimen was estimated by comparing their ELISA-OD with those of serially diluted standard positive specimens with predetermined endpoint titer (Igarashi *et al.*, 1981; Morita *et al.*, 1982).

Infectivity assay of dengue viruses: Microfocus assay method of Okuno *et al.* (1985) was used with modifications. Virus specimens were serially diluted in virus diluent (cell growth medium from which serum concentration was reduced to 5%). Growth medium was removed from BHK21 cell cultures on 96 well microplate (Nunc, Denmark) using 100 μl/well. Fifty microliter volume of the diluted specimens were inoculated into replicate wells, and adsorption was carried out for 2 hours at 37℃ in 5% CO_2−atmosphere spreading the inoculum over cell sheet every 30 minutes. The cell sheets were then covered by 100 μl/well of the overlay medium (0.5% methyl cellulose 4000 in the maintenance medium). The plate was incubated as above for 3 days in the case of D2 and D4, 4 days in the case of D3, and 5 days in the case of D1, respectively. Foci of BHK21 cells carrying intracellular viral antigen were visualized by immunoperoxidase staining, using incubation at room temperature and PBS for rinsing and diluent. After the overlay medium was removed, the plate was rinsed, and the cells were fixed with 5% formaldehyde for 20 minutes. After rinsing, the plate was successively reacted with DHF patient's serum at 1:500 and HRPO-conjugated anti-human IgG (Cappel, USA) at 1:1,000 dilution, for 1 hour each. The HRPO reaction was carried out for 5 minutes using the substrate solution containing 0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride and 0.02% H_2O_2. The plate was rinsed with tap water and foci of brown colored cells were counted using a magnifying lens. The infectivity titer was expressed as focus forming units (FFU)/ml.

RESULTS

In preliminary experiments, dengue virus was inoculated into replicate cultures of C6/36 cells grown in rubber-stoppered bottles, and the specimens were collected after 1 and 2 weeks incubation at 28℃, 32℃ or 37℃. The sandwich ELISA showed that the amount of D2 and D3 antigen produced in the infected culture fluid was significantly elevated when the cells were incubated at 32℃ or 37℃ than at 28℃. In contrast, D1 antigen production in the infected fluid was not so much elevated by increasing the incubation temperature. While almost undetectable amount of D4 antigen was produced under any incubation temperatures examined although infective virus was detected in the culture supernatant. These preliminary experiments also showed that the amount of cell associated dengue antigen or infectivity was

not greater than the amount found in the infected fluid.

Daily production of dengue antigens and infectivity in the infected C6/36 cell culture fluid was examined at 3 different incubation temperatures. Replicate cultures of C6/36 cells on 24-well plates were inoculated with the seed virus and incubated at 28℃, 32℃ or 37℃. Infected culture fluid was collected daily from different wells until 7 days after inoculation, and assayed for HA, ELISA and infectivity as described in the Materials and Methods. The results summarized in Fig. 1 confirmed the data obtained in the preliminary experiments: (1)

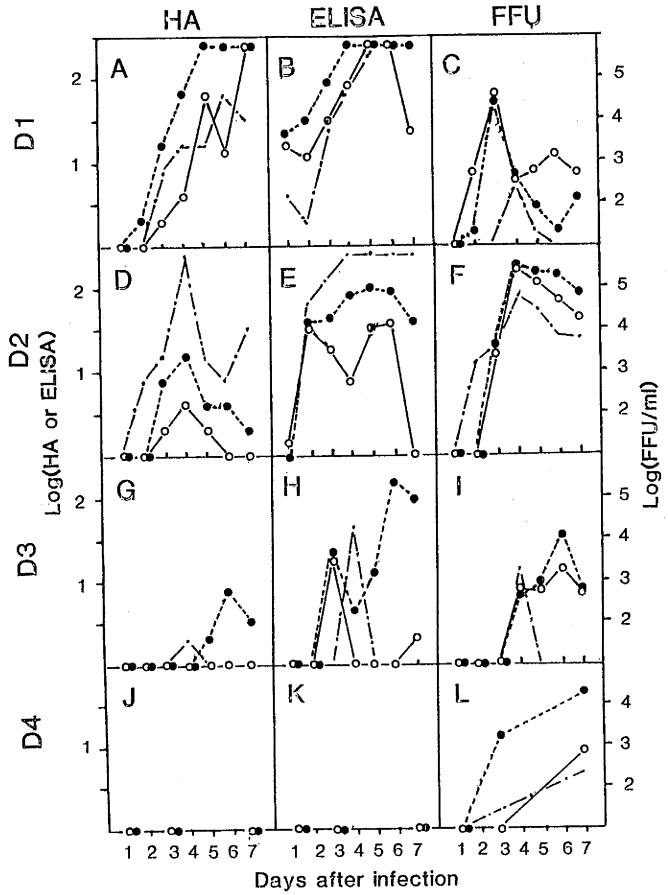

Fig. 1. Production of dengue viral HA, ELISA antigen and infectivity in the infected C6/36 cell culture fluid at 3 different temperatures.

Replicate wells of C6/36 cultures on 3 different 24 well plates were inoculated with dengue viruses (0.1ml/well). After 2 hours adsorption at 28℃, the cells were covered by maintenance medium (1 ml/well) and incubated at 28℃ (○——○), 32℃ (●·····●), or 37℃ (—•—•—) in 5% CO_2—atmosphere. Infected fluid was collected daily from different wells, and HA, ELISA antigen and infectivity (FFU) titers were measured as described in the Text.

Panels A: D1-HA, B: D1-ELISA, C: D1-FFU, D: D2-HA, E: D2-ELISA, F: D2-FFU, G: D3-HA, H: D3-ELISA. I: D3-FFU, J: D4-HA, K: D4-ELISA and L: D4-FFU.

enhanced antigen production of D2 by elevated incubation temperature, (2) similar but less enhancement of D3 antigen production at 32℃ than 28℃, (3) almost similar maximum antigen production of D1 at elevated temperatures, and (4) undetectable levels of D4 antigen production at all temperatures examined. Besides in the case of D1, the maximum titer of HA or ELISA were reached 1−2 days earlier at 32℃ than at 28℃, although higher temperature at 37℃ did not provide beneficial effect on the maximal level of D1 antigen production. It contrast to the enhancement of D2 (and less for D3) antigen production at elevated incubation temperature, infectivity produced in the infected fluid was almost similar at 3 temperatures, or even less at 37℃ than 28℃. It is worthwhile to notice that even in the presence of comparable infectivity of D4 as D1 or D3, D4 antigen was not detectable in the infected culture fluid.

Discussion

The data in this study showed different response among 4 different serotypes of dengue viruses in terms of antigen production in the infected C6/36 cell culture fluid at elevated temperature. It appears interesting to clarify whether the observed difference is an intrinsic characteristic to each serotype, or depends on particular strain among each serotype of dengue viruses. This question should be examined using multiple strains of the same serotype, for example D2, isolated from different sources or patients with different clinical manifestations. Discrepancy between the antigen and infectivity titers in the infected culture fluid at elevated temperature could probably be due to different heat-stability of dengue antigens and infectivity. That is, at higher temperature, amount of dengue virion produced in the infected culture fluid was increased, but their infectivity was more quickly inactivated than their antigens.

It should also be determined whether the apparent enhancement of D2 (and less for D3) antigen production in the infected fluid at elevated temperatures is the result of enhanced release of the intracellular accumulated antigens or enhanced synthesis of viral macromolecules. This question shoud be examined by quantitative assay on intracellular as well as extracellular viral antigens and viral RNA.

Regardless of these possibilities, the observed results enable to produce higher titer of D2 and D3 antigens in the infected C6/36 cell culture fluids, which could be utilized for routine diagnostic tests or epidemiological surveillance on dengue virus infection.

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