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Antigen Production of Dengue Type 4 Virus strains in

Aedes albopictus clone C6/36 and Vero Cell Cultures

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Abstract: Antigen production of seven dengue type 4 virus strains in *Aedes albopictus* clone C6/36 cells at 28℃ and in Vero cells at 37℃ was studied. These seven strains could roughly be divided into three different entities according to their pattern of antigen production. The first entity of prototype strain (H-241) produced ELISA antigen in vero cells at 37℃, whereas its antigen production was undetectable in C6/36 cells upto day 11 post infection. The second entity, strain No. 17 (1978 − Sri Lankan strain) revealed the reverse pattern : high antigen titer in C6/36 cells and undetectable titer in vero cells. The remaining five strains belonged to the third entity, in which viral antigen production was found in both cell lines to similar titers.

Key words: Dengue type 4 virus antigens, *Aedes albopictus* clone C6/36 cells, Vero cells

INTRODUCTION

Dengue is a human disease of global significance. Up to 100 million cases of dengue infection per year worldwide can be estimated from available data if one assumes there is an average annual infection rate of 10% for endemic areas, with most susceptible hosts being children (Halstead, 1980 & 1988; Sangkawibha *et al.,* 1984). In terms of total number of infections, total number of sick persons and total number of deaths, dengue is by far the most important human mosquito-borne viral pathogen (Haltsead, 1993). Dengue virus infection occurs in endemo-epidemic pattern in many tropical countries especially those in the Southeast Asian region. Awareness of its prevalence and early clinical diagnosis, successively followed by confirmed laboratory diagnosis is indispensable for proper clinical case management. Precise information on the disease agents obtained by the laboratory diagnosis could also be used as indicators for epidemiological surveillance and formulating the control strategy to prevent its outbreak (Igarashi, 1994). Serological diagnoses are most widely applicable in many laboratories, especially those in the developing countries. Currently available serodiagnoses include the hemagglutination-inhibition test (HI) test (Clark and Casals, 1958; Shope and Sather, 1979; World Health Organization, 1986), and IgM-enzyme linked immunosorbent assay (IgM-ELISA) tech-

nique (Burke, 1983; Bundo and Igarashi, 1985; Lam *et al.,* 1987; Innis *et al.,* 1989). Assay antigens prepared from infected suckling mouse brains have been used for majority of these tests, but it requires maintenance of mouse colonies, tedious extraction procedures with sucrose-acetone or acetone-ether, and high running cost. Hence, the production of high titered dengue antigens in the infected *Aedes albopictus* clone C6/36 cell line (Igarashi, 1978) has been tried by some researchers (Soe Thein *et al.,* 1979; Cardosa and Zuraini, 1991; Mohamed *et al.,* 1995), in order to utilize it in the routine diagnosis and epidemiological surveillance. In these studies dengue type 4 viral antigen was most difficult to produce among four serotypes of dengue virus.

Our recent study is one of the above entities to observe the pattern of viral antigen production in C6/36 cells at 28℃ and in vero cells at 37℃ among seven strains of dengue type 4 viruses originated from South and Southeast Asian region.

<div align="center">MATERIALS AND METHODS</div>

Virus strains

Dengue type 4 virus strains used in this study were listed in Table 1. All strains were grown in Aedes albopictus clone C6/36 cells at 28℃ for one week, and the seed viruses were stored in aliquots at −80℃ until use.

Cell cultures

Aedes 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%

Table 1 Dengue type 4 virus strains used in this study

Strains	Country	Year	Diagnosis
H-241	Prototype	—	—
SI-YO SMB 10	Thailand	1978	DHF
No-17	Sri Lanka	1978	—
PhMH 2123-83	Philippines	1983	—
ThNH-44/93	Thailand	1993	DHF grade I
ThNH-102/93	Thailand	1993	DHF grade III (DSS)
ThNH-p21/93	Thailand	1993	DF

(1) DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome

(2) Origin of each strain

 H-241, the prototype strain, was obtained from National Institute of Health, Japan

 Sl-YO SMB10 was isolated from patient's serum of laboratory infection by strain No. 124 from Thailand, and passaged 10 times in SMB. (obtained from Research Institute for Microbial Diseases of Osaka University)

 No-17 was isolated by Dr. Rosen and obtained from Dr. Inoue, National Institute of Health, Japan

 PhMH 2123-83 was isolated from patient's serum in Manila, Philippines, in 1983

 ThNH-44/93, ThNH-102/93 and ThNH-p21/93 were isolated by Dr. Igarashi on his overseas research work in Nakhon Phanom, Thailand, in 1993

heat-inactivated fetal calf serum (FCS). The cells were grown in multiwell dishes in 5% CO_2 atmosphere. Vero cells were grown at 37℃ using the same medium as C6/36 cells.

Virus inoculation and specimen collection

Growth medium was removed from C6/36 and vero cell cultures and seed viruses were inoculated according to the set up protocol. The inoculum size was 0.1 ml/well on 24-well plates. Adsorption was carried out for 2 hours at respective temperatures (28℃ for C6/36 cells and 37℃ for vero cells) spreading the inoculum over cell sheet every 30 minutes. After adsorption, the cells were covered by the maintenance media (the same growth medium from which FCS concentration was reduced to 2%) using 1ml/well on 24 well plates. The infected cells were incubated at 28℃ for C6/36 cells and 37℃ for vero cells, in 5% CO_2 atmosphere until day 11 after infection. The culture fluids infected with each strain of dengue 4 viruses were collected daily for 12 consecutive days (day 0 to day 11 post infection). The specimens were stored at 4℃ for antigen assay.

ELISA antigen assay

Micro sandwich method of Voller et al. (1976) was followed using incubation at room temperature for 1 hour and reagent volume of 100μl/well for each reaction step. A 96-well flat bottom plate (Nunc, Denmark) was sensitized overnight by anti-flavivirus IgG (20μg/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, containing 0.01% NaN_3. The IgG was purified from high titered DHF patients' sera 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 specimens, positive control specimen (dengue 2 standard antigen) in serial 2fold dilution steps in the diluent (same as the maintenance medium), and negative control of mock-infected culture fluid were distributed in the wells. After washing as mentioned previously, 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 adding the substrate solution of 0.5mg/ml o-phenylenediamine dihydrochloride (OPD) and 0.02% H_2O_2 in 0.05 M citrate phosphate buffer, pH 5. After 30 minutes-1 hour incubation in the dark at room temperature, the reaction was stopped by adding 100μl of 1N sulphuric acid to each well. The OD_{492} on each well was measured by an ELISA reader using 620nm 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).

RESULTS

Fig. 1 shows the level of dengue type 4 antigen production in two different cell culture systems (28℃ for C6/36 cells and 37℃ for vero cells). According to these results, seven dengue type 4 virus strains studied could roughly be divided into three different entities. The first entity of prototype strain (H-241) produced ELISA antigen in vero cells at 37℃, whereas its antigen production was undetectable in C6/36 cells upto day 11 post infection [Fig. 1 (A)].
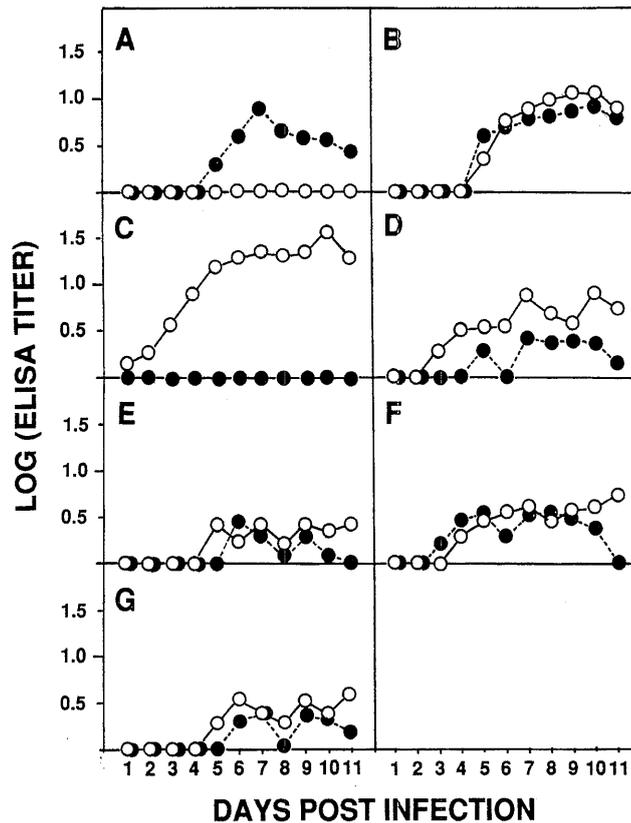
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Fig. 1. Dengue type 4 virus antigen production in C6/36 cells at 28℃ and vero cells at
37℃ incubation temperatures.
C6/36 cell and vero cell cultures on different 24 well plates were inoculated with
seven different strains of dengue 4 viruses (0.1ml/well). After 2 hours adsorption
at respective temperatures, the wells were covered by maintenance medium (1
ml/well). C6/36 cells were incubated at 28℃ (○······○), and vero cells at
37℃(●······●) in 5% CO_2 atmosphere. Infected fluid was collected daily from
different wells upto day 11 post infection. Antigen ELISA titer was measured as
described in the Text.
Panels A: H-241 (prototype), B: SI-YO SMB10, C: No.17,
        D: ThNH-44/93, E: ThNH-102/93, F: ThNH-p21/93
        and G: PhMH 2123-83.
Details of the seven virus strains used in this study were mentioned in the text
and Table 1.

The second entity, strain No. 17 (1978-Sri Lankan strain) revealed the reverse pattern to
prototype strain, since it produced high antigen titer in C6/36 cells and undetectable antigen in
vero cells [Fig. 1 (C)]. The remaining five strains belonged to the third entity, in which viral an-
tigen production was found in both cell lines, and the antigen titer produced was more or less the
same for both cell lines except slightly higher titer in C6/36 cells than in vero cells was observed
in ThNH-44/93 strain [Fig. 1 (D)]. The level of antigen production was rather low in the third
group.

In the preliminary experiments, dengue type 4 virus strains H-241 (prototype), SI-YO SMB10 (Thai strain) and strain No-17 (Sri Lankan strain) were also inoculated into BHK 21 cells in multiwell plates and grown at 37℃ until day 11 post infection. Infected culture fluid of these strains were harvested daily and measured the amount of antigen production by sandwich ELISA. The results showed that undetectable amount of D4 antigen was produced under the conditions examined (data not shown). The preliminary experiments on H-241 strain also showed that the amount of cell associated dengue antigen was more or less the same to that found in the infected fluid.

DISCUSSION

Attempts to explore the availability of cell culture derived high titered dengue antigens have been made by several workers. Buckley and Srihongse (1963) reported the production of dengue hemagglutinin in infected HeLa cells, but this hemagglutinin has not been used instead of standard SMB antigen in routine tests. LLC-MK$_2$ cell cultures infected with dengue viruses were found to produce sufficiently high titers of hemagglutinin in the culture fluid for use as diagnostic antigens under specified conditions (Rosen, personal communication; Inoue, personal communication).

Soe Thein *et al.* (1979) showed that dengue type 1 virus antigen was produced in significant titer in culture fluids of C6/36 cells, which can be used in routine diagnosis or epidemiological surveillance. Similar attempts to produce other serotypes of dengue antigen were not successful at that time. Mohamed *et al.* (1995) reported that the amount of dengue type 2 and type 3 viral antigens produced in the C6/36 cells culture fluid was increased by elevating the incubation temperature of infected cells to 32℃ or 37℃. The maximum titer of type 1 dengue virus antigen was not significantly elevated although they reached maximum titer 1−2days earlier. Type 4 dengue viral antigen was almost undetectable under the conditions examined (incubation temperature at 28℃, 32℃ and 37℃), even though its infective virus was produced in the culture fluid.

Another series of experiments performed in our laboratory showed that increase in the incubation temperature of C3/36 cells from its optimal growth temperature of 28℃ to 32℃ and 37℃ resulted in the increased viral RNA synthesis together with antigen production of dengue type 2 virus. (Mangada *et al.,* data submitted for publication).

In this study, we have measured the level of dengue 4 antigen production in the culture fluid of C6/36 cells at 28℃ and vero cells at 37℃ by sandwich ELISA method. Absence of detectable antigen in the infected C6/36 culture fluid of prototype strain for the examined period (day 0 to day 11 post infection) was in agreement with the findings of Mohamed *et al.,* (1995). It seems that there was a strain specificity on the antigen production. Prototype strain showed better antigen production in vero cells at 37℃, reaching its peak on day 7 post infection. Based upon our findings, it is advisable to produce the high titered antigen for dengue 4 prototype strain H-241 in vero cell line at 37℃.

On the other hand, strain No-17 revealed highest antigen titer (upto 36 ELISA units) among all the strains examined, which was detected in C6/36 cells at 28℃ on day 10 post infection. This

Sri Lankan strain can possibly be used as an alternative source for high titered dengue-4 antigen production in C6/36 cell line instead of growing prototype strain in vero cell line. Interesting point is that strain No-17 did not produce detectable antigen in vero cell line. It might be an intrinsic characteristics of that specific virus strain.

Another interesting fact is that antigen production of various strains of viruses has no relationship with its disease severity. In other words, DSS strain (ThNH-102/93) did not produce higher titer of antigen than other DHF and DF strains (Table. 1 and Fig. 1).

Our recent study provided the information essential for the selection of appropriate cell line and incubation temperature for the production of high titered dengue 4 antigen, which could possibly replace the conventional SMB antigen for the diagnostic purposes.

Acknowledgement

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