Tropical Medicine, 25 (3), 149–154, September, 1983

Virological and Epidemiological Studies on Encephalitis in Chiang Mai Area, Thailand, in the Year of 1982

III. Virus isolation from clinical materials

Akira IGARASHI

Department of Virology, Institute for Tropical Medicine, Nagasaki University

Pien CHIOWANICH

Department of Pediatrics, Faculty of Medicine Chiang Mai University, Chiang Mai, Thailand

Pranee LEECHANACHAI and Jiraporn SUPAWADEE

Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Abstract: Japanese encephalitis (JE) virus was isolated from one out of 3 postmortem brain materials taken as necropsy specimens, while 11 strains of dengue viruses were isolated from peripheral blood of 9 dengue hemorrhagic fever (DHF), 1 encephalitis and 1 aseptic meningitis patients, during study period from July 19 to August 17, 1982. Typing by monoclones revealed that 8 strains were type 1, 2 were type 2, and 1 was type 3 virus, respectively. Isolation of dengue viruses from encephalitis and meningitis cases appears to indicate the presence of dengue encephalopathy.

Key words: Encephalitis, Thailand, Virus isolation.

INTRODUCTION

Since 1969, many encephalitis cases have been reported every year in Thailand, mainly from Northern and Northeastern regions. Most of the cases were observed during rainy season of June to August, although small numbers were found all year round (Grossman, *et al.*, 1973; Statistics of the Ministry of Public Health of Thailand). As described in the accompanying paper (Igarashi *et al.*, 1983), we attempted to isolate viruses from fatal encephalitis cases and peripheral blood of dengue hemorrhagic fever (DHF) and other diseases, because dengue infection was known to coexist in this area during rainy season (Grossman *et al.*, 1973b). The method of virus isolation was

Received for pablication, September 26, 1983.
Contribution No. 1374 from the Institute for Tropical Medicine, Nagasaki University.

to inoculate test materials to *Aedes albopictus*, clone C6/36, cells. The clone was developed as a sensitive clone to dengue and ckikungunya viruses (Igarashi, 1978) from Singh's *Ae. albopictus* cell line (Singh, 1976). The validity of the method to isolate dengue viruses was shown by Tesh (1979) and applied to clinical materials by several investigators (Fukunaga *et al.*, 1980; Rojanasuphot *et al.*, 1981; Igarashi *et al.*, 1982). The senior author with his colleagues demonstrated the validity of the method to isolate Japanese encephalitis (JE) virus from mosquitoes, and postmortem brains in Japan (Igarashi *et al.*, 1981a; b). Recent development of monoclonal antibodies specific to each of the 4 types of dengue viruses (Henchal *et al.*, 1982) made identification and typing of dengue viruses quite easy and rapid to be performed in Chiang Mai. This paper describes isolation of JE and dengue viruses from clinical materials collected during our study period from July 19 to August 17, 1982.

MATERIALS AND METHODS

Cells: Clone C6/36 of Singh's *Ae. albopictus* cell line was grown as described previously (Igarashi, 1978) with cell growth medium of 10% heat-inactivated fetal calf serum in Eagle's medium supplemented with 0.2 mM each of nonessential amino acids (Eagle, 1959). The cells were seeded in 16×126 mm test tubes at a density of 10^5 cells/ml, using 2 ml/tube. After 3 days of incubation at room temperature (25−30°C), the cells formed almost monolayers. In the case of 8-chamber slides (Lab-Tek 4838, Miles, Ill. USA), each well received 0.4 ml of the cell suspension at the same density. The slides were incubated at room temperature for 3 days in a sealed box with sodium bicarbonate-saturated water at the bottom.

Virus isolation procedures: The method is similar to those described for mosquito specimens (Igarashi *et al.*, 1981a). Postmortem brains or livers were homogenized in PBS (phosphate-buffered saline, pH 7.2) containing 0.2% of bovine plasma albumin (Armour, Faction V), to make 5-10% suspension, and centrifuged at 2500 rpm for 15 min. The rupernatant was filtered through Millipore type HA filter and one-tenth ml of the filtrate was inoculated to tube culture of C6/36 cells after removal of the cell growth medium. The adsorption was carried out at room temperature for 2 hours and the cells were refed with 2 ml/tube of the maintenance medium (cell growth medium from which serum concentration was reduced to 2%). After 7 days of incubation at room temperature, the presence of infective virus in the medium was detected by inoculation to C6/36 cells grown on 8-chamber slides. The slides were harvested 3 days later in order to detect intracellular viral antigens by the immunoperoxidase staining (Okuno *et al.*, 1977). Antiviral rabbit sera used in this screening were raised against JE, chikungunya, Sindbis, and Getah viruses, by 2 intramuscular inoculations with 4 weeks interval of purified virus mixed with an equal volume of Freund's complete adjuvant. The rabbits were bled 1 we-

ek after the second immunization. These sera were used at 1:1000 dilution in the staining. Specimens which showed positive staining by anti-JE serum was further tested by type-specific anti-dengue monoclonal antibodies, which were kindly supplied by Dr. T. Monath, CDC, USA. The monoclones were diluted 1:1000 and reacted to C6/36 cells inoculated with test materials and fixed with acetone. The cells were then reacted with anti-mouse IgG rabbit serum at 1:1000 dilution, followed by similar proceudres of immunoperoxidase ataining (Okuno *et al.*, 1977). This procedure gave clear-cut differentiation among each of the 4 prototypes of dengue viruses and was used in the studies in Indonesia (Igarashi *et al.*, 1982). The specimen which was stained by anti-JE serum but not by dengue monoclones was examined by the neutralization test against anti-JE serum. In the case of serum specimens, the procedure described for the studies in Indonesia (Igarashi *et al.*, 1982) and for swine sera in Japan (Igarashi *et al.*, 1981c) was used. Serum specimens of 0.1 ml volume was inoculated to tube culture of C6/36 cells without removing growth medium. After overnight incubation, old medium was removed and the cells were refed with fresh maintenance medium. The cells were further incubated at room temperature for 6 more days and the presence of the virus was screened as described above.

RESULTS

Total numbers of specimens tested for virus isolation was 177 sera, 3 brains, and 1 liver specimens from hospitalized patients. Brains and liver specimens were obtained as necropsy materials by needle puncture. As controls, 50 sera from healthy adults were used which were collected at a Blood Bank in Chiang Mai. Table 1 summarizes the result of positive virus isolation from these specimens, as arranged by the date. Only one JE virus was isolated from postmortem brain of patient (P-19) who died on July 26, 8 days after onset of encephalitis. His paired sera showed typical response of acute JE virus infection as shown in the following paper (Fujita *et al.*, 1983), and the isolated virus was neutralized by anti-JE serum. On the other hand, total of 11 strains of dengue viruses were isolated from hospitalized patients' sera. Nine of them were obtained from DHF patients, however, one of the remaining 2 was from encephalitis, and the other from aseptic meningitis cases, B-8 and P-9, respectively. The B-8 case was 67 years old female who developed encephalitis on July 12 and dengue virus type 1 was isolated from serum specimen collected on August 2, 21 days after the onset. Since IgM-ELISA and HI against JE were negative, it was hard to consider that she was first infected with JE virus showing encephalitis, followed by dengue virus infection which produced viremia. Another meningitis patient, P-6, was an 11 years old male with his serum taken on July 21, 2 days after the onset. These 2 cases might represent dengue encephalopathy (Sumarmo *et al.*, 1978). Typing of dengue virus isolates was done by monoclonal antibodies and the result showed that 8 were type 1, 2 were type 2, and 1

Table 1. Isolation of viruses from clinical specimens in Chiang Mai, 1982

Code	Age and sex	Clinical diagnosis	Virus isolation				Serodiagnosis			
			Date of onset	Specimen taken	Date (days of illness)	Virus	HI		ELISA	
							JE	D1	IgG	IgM
B8	67 F	Encephalitis	Jul 12	Blood	Aug 2(21)	D1	−	+	+	−
P6	22 F	DHF*	unknown	Blood	Jul 13	D2	+	+	+	−
P9	11M	Aseptic meningitis	Jul 19	Blood	Jul 21 (2)	D2	−	−	−	−**
P13	1 F	DHF	Jul 19	Blood	Jul 23 (4)	D1	−	−	−	−
P15	13 F	DHF	Jul 18	Blood	Jul 19 (1)	D3	−	−	−	−
P19	5M	Encephalitis	Jul 18	Brain	Jul 26 (8)	JE	+	−	−	+
P56	5M	DHF	Jul 28	Blood	Aug 2 (5)	D1	+	−	−	−**
P57	16M	DHF	Jul 29	Blood	Aug 2 (4)	D1	+	+	+	−
P59	6M	DHF	Jul 28	Blood	Aug 2 (5)	D1	+	+	+	−
P68	40M	DHF	Jul 29	Blood	Jul 30 (1)	D1	+	+	+	−
P76	5M	DHF	Jul 27	Blood	Aug 3 (7)	D1	−	+	+	−
P77	31M	DHF	Aug 1	Blood	Aug 3 (2)	D1	+	+	+	−

* DHF: dengue hemorrhagic fever
** single serum specimen
*** HI(+): 4-fold or more rise in paired sera, or more than 640 in single serum
ELISA (+): 4-fold or more rise in paired sera, or more than 8000 of IgG or more than 400 in IgM in single serum specimen

was type 3 dengue virus. Therefore, principal type of dengue viruses circulating in Chiang Mai during our study period from July 19 to August 17 was type 1 virus. Two cases, P-13 and P-15, with positive virus isolation of dengue virus type 1 and type 3, respectively, did not show any antibody response either by the HI or the ELISA. Thus, these 2 DHF cases were shown to be infected with dengue viruses only by virus isolation. The timing of bleeding was probably too early to detect primary type of antibody response in these 2 cases. Fifty normal human sera and 1 post-mortem liver did not give any virus isolates.

DISCUSSION

The result of virus isolation showed that dengue viruses were relatively easy to isolate from DHF patients, while JE virus was not isolated from peripheral blood. The result agrees with the fact that humans do not show viremia of JE virus. It may be interesting to examine type 1 dengue virus which was isolated from an encephalitis patient, B-8. If this virus is different from other type 1 dengue viruses isolated in this

study, in terms of its genetic constitution, it may give some indication that pathogenicity of the virus is depending on the virus gene. So far our result is the first report of dengue virus isolation in Chiang Mai Area. None of the 50 normal human sera yielded dengue virus, indicating that inapparent infection with dengue viruses in adults with viremia is a rare event. Previous observations in Indonesia by senior author and his colleagues showed that mild febrile cases often exhibit viremia of dengue viruses, and would probably play an important role in the maintenance and circulation of the virus in endemic and epidemic areas (Igarashi *et al.*, 1982).

REFERENCES

1) Eagle, H. (1959): Amino acid metabolism in mamalian cell cultures. Science, 130, 432−437.
2) Fujita, N., Igarashi, A., Ogata, T., Supawadee, J., Peerakome, S., Leechanachai, P., Panasampol, K., Chanyasanha, C., & Chatiyanonda, K. (1983): Virological and epidemiological studies on encephalitis in Chiang Mai Area, Thailand, in the year of 1982. Ⅳ. Serological examination on hospitalized patients. Trop. Med., 25, 155−164.
3) Fukunaga, T., Okuno, Y., Srisupaluck, S., Auvanich, W., Rojanasuphot, S., Sangkawibha, N., Kasemsarn, P., & Dharakul, C. (1980): Serological and virological studies on patients with dengue hemorrhagic fever (DHF) in Chanthaburi Province, Thailand. Ⅱ. Serological characteristics of viruses isolated from DHF patients using a clone of Singh's *Aedes albopictus* cells. Biken J., 23, 123−133.
4) Grossman, R. A., Edelman, R., Chiewanich, P., Voodhikul, P., & Siriwan, C. (1973a): Study of Japanese encephalitis virus in Chiangmai Valley, Thailand. Ⅱ. Human clinical infections. Amer. J. Epidemiol., 98, 121−132.
5) Grossman, R. A., Edelman, R., Willhight, M., Pantuwatana, S., & Udomsakdhi, S. (1973b): Study of Japanese encephalitis virus in Chiangmai Valley, Thailand. Ⅲ. Human seroepidemiology and inapparent infections. Amer. J. Epidemiol., 98, 133−149.
6) Henchal, E. A., Gentry, M. K., McCown, J. M., & Brandt, W. E. (1982): Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by immunofluorescence. Amer. J. Trop. Med. Hyg., 31, 830−836.
7) Igarashi, A. (1978): Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol., 40, 531−544.
8) Igarashi, A., Buei, K., Ueba, N., Yoshida, M., Ito, S., Nakamura, H., Sasao, F., & Fukai, K. (1981a): Isolation of viruses from female *Culex tritaeniorhynchus* in *Aedes albopictus* cell cultures. Amer. J. Trop. Med. Hyg., 30, 449−460.
9) Igarashi, A., Makino, Y., Matsuo, S., Bundo, K., Matsuo, R., Higashi, F., Tamoto, H., & Kuwatsuka, M. (1981b): Isolation of Japanese encephalitis and Getah viruses by *Aedes albopictus* clone C6/36 cells and by suckling mouse brain inoculation in Nagasaki, 1980. Trop. Med., 23, 69−78.
10) Igarashi, A., Morita, K., Bundo, K., Matsuo, S., Hayashi, K., Matsuo, R., Harada. T., Tamoto, H., & Kuwatsuka, M. (1981c): Isolation of Japanese encephalitis and Getah viruses, from *Culex tritaeniorhynchus* and slaughtered swine blood using *Aedes albopictus* clone C6/36 cells

in Nagasaki, 1981. Trop. Med., 23, 177−187.
11) Igarashi, A., Fujita, N., Okuno, Y., Oda, T., Funahara, Y., Shirahata, A., Ikeuchi, H., Hotta, S., Wiharta, A. S., Sumarmo, & Sujudi. (1982). Isolation of dengue viruses from patients with dengue hemorrhagic fever (DHF) and those with fever of unknown origin (FUO) in Jakarta, Indonesia, in the years of 1981 and 1982. ICMR Annals, Kobe Univ., 2, 7−17.
12) Igarashi, A., Srisukrit, A., & Tuchinda, P. (1983): Virological and epidemiological studies on encephalitis in Chiang Mai Area, Thailand in the year of 1982. Ⅰ. Introduction and study design. Trop. Med., 25, 128−138.
13) Okuno, Y., Sasao, F., Fukunaga, T., & Fukai, K. (1977): An application of PAP (peroxidase-anti-peroxidase) staining technique for the rapid titration of dengue virus type 4 infectivity. Biken J., 20, 29−33.
14) Singh, K. R. P. (1967): Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr. Sci., 36, 506−508.
15) Sumarmo, Wulur, H., Jahja, E., Gubler, D. J., Sutokenggolo, T. S., Sulianti Saroso, J. (1978): Encephalopathy associated with dengue infection. Lancet, 1, 449−450.
16) Rojanasuphot, S., Chanyasanha, C., Ahandrik, S., Chatiyanonda, K., Igarashi, A., & Inoue, S. (1981): Isolation and identification of dengue viruses by combined use of C6/36 cells and the immune adherence hemagglutination test. Japan. J. Med. Sci. Biol., 34, 375−379.
17) Tesh, R. B. (1979): A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Amer. J. Trop. Med. Hyg., 58, 1053−1059.

1982年タイ国チェンマイ地区における脳炎のウイルス学的疫学的調査. Ⅲ. 臨床材料からのウイルス分離

五十嵐 章（長崎大学熱帯医学研究所ウイルス学部門）
Pien CHIOWANICH（タイ国チェンマイ大学医学部小児科学教室）
Pranee LEECHANACHAI, Jiraporn SUPAWADEE（タイ国チェンマイ大学医学部微生物学教室）

1982年7月19日から8月17日までの調査期間中，3例の死亡した脳炎患者中1検体から1株の日本脳炎ウイルスが分離された．一方患者末梢血177検体からデングウイルスは11株分離され，単一クローン抗体による型同定で8株は1型，2株は2型，1株は3型である事が判明した．脳炎あるいは髄膜炎と診断された患者からデングウイルスが分離されたことはデング脳症の存在を示すものと考えられる．

熱帯医学 第25巻 第3号，149−154頁，1983年9月