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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

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SUMMARY Four strains of dengue (DEN) virus were isolated using C6/36 cells, a clone of Singh's *Aedes albopictus* cells, from acute phase sera of DHF patients in Thailand in 1978.

The isolates grown and passaged in C6/36 cells were not neutralized appreciably by standard rabbit antisera against each of the 4 types of DEN virus, but their serotypes could be identified by complement fixation (CF) tests. After the 4th or 10th passage in suckling mouse brain (SMB), however, three of the four strains showed type-specific reactions in neutralization (N) tests against the standard antisera.

In cases of secondary infection, the highest N titer in the convalescent phase sera was demonstrated not against the isolated serotype, but against the serotype considered to have caused primary infection. Moreover in cases of secondary infection, the convalescent phase sera showed a significantly lower N titer against the newly isolated strain derived from the same patient than against the corresponding

prototype standard virus. This phenomenon was not observed in a single case of primary infection.

INTRODUCTION

In 1958 an epidemic of DHF occurred in and near Bangkok (Hammon et al., 1960) and was provisionally designated as Thai hemorrhagic fever (Hammon and Sather, 1964). Since then DHF has been one of the most important virus diseases in Thailand, because of its high infection rate in children, sometimes causing shock and death (Halstead, 1966). During the 1960's, DHF was largely confined to large cities, but now it is increasingly involving provincial cities and towns and spreading to most portions of the country (WHO Report, 1978).

The pathogenesis of DHF is still unclear even though it is more than 20 years since DEN and chikungunya (CHIK) viruses were first isolated from DHF patients (Hammon et al., 1960). However, it seems safe to assert that the pathogenesis of DHF is much more complicated than those of other flavivirus infections such as yellow fever (YF) or Japanese encephalitis (JE), because DEN virus has at least 4 serotypes, while YF and JE viruses have only one, without secondary manifestation of the diseases.

For an understanding of the epidemiological principles of DHF, it is essential to obtain adequate information on the biological and ecological characteristics of DEN viruses in nature. It is known that the viruses are transmitted by *Stegomiya* mosquitoes, such as *Aedes aegypti* or *Aedes albopictus* (Clarke and Casals, 1965). Consequently, it seems better and more natural to use *Aedes* mosquito cells than commonly used newborn mice or mammalian cells as hosts in DEN virus isolation. Therefore, we tried to isolate DEN viruses using a cloned C6/36 cell line, which was isolated from Singh's *Aedes albopictus* cells and was proved to be sensitive to DEN and CHIK viruses (Igarashi, 1978).

This paper describes the serological characteristics of the isolated DEN viruses and the results of N tests on paired sera of DHF patients from which viruses were isolated.

MATERIALS AND METHODS

1. *Cells*

1) For virus isolation

The C6/36 clone of *A. albopictus* cells was supplied from Dr. Igarashi of our Department. Cells were grown in 2-ounce bottles or in tubes at 28°C in cell growth medium consisting of 10% fetal calf serum (FCS) in Eagle's medium (Eagle, 1959) supplemented with 0.2 m*M* concentrations of each non-essential amino acid.

2) For virus infectivity assay and the N test

BHK21 cells were cultured in growth medium consisting of 10% FCS in Eagle's medium at 37°C in Lab-Tek 8-chamber slides (Miles, Ill., USA) in a CO_2-incubator for one day before use.

2. *Virus isolation*

All blood specimens except No. SI-8 were collected from DHF patients in the Department of Pediatrics, Prapokklao Hospital, Chanthaburi, located about 300 km south-east of Bangkok. Specimen No. SI-8 was collected in Bangkok. The serum specimens collected in Chanthaburi were stored in the freezing compartment of a refrigerator for 1–2 weeks before being transported in an ice box by car to the Virus Research Institute in Bangkok.

For serodiagnosis, hemagglutination inhibition (HI) tests were performed in the Virus Section of the Public Health Laboratory, Chanthaburi. Acute phase sera with low or undetectable HI titers against DEN antigens were chosen as materials for virus isolation. Samples of 0.2 ml of 2–5 fold diluted sera were inoculated onto C6/36 cells grown in 2-ounce bottles. After adsorption for 2 h, the cells were covered with 5 ml of maintenance medium, which was the same as growth medium except that the FCS concentration was lower (2%). The culture fluids of C6/36 cells with CPE of syncytium

formation were harvested and kept in a Revco freezer at −70°C for further infectivity assay and passages.

3. *Anti-DEN sera*

1) Standard anti-DEN rabbit sera

Prototype viruses of DEN 1 (Hawaiian strain), DEN 2 (New Guinea B strain), DEN 3 (H-87 strain) and DEN 4 (H-241 strain) were used as immunogens. Rabbits were given 2 intramuscular injections of virus in Freund's complete adjuvant with an interval of one month between injections. The materials injected were partially purified virus samples prepared from infected SMB by protamine sulfate clarification and ultracentrifugation (Smith et al., 1970). Rabbits were bled 10 days after the last injection.

2) Anti-DEN mouse sera

Prototype viruses and some isolates were used for immunization. Adult mice were injected with 0.5 ml of the supernatants of 10% homogenates prepared from infected SMB. Injections were given intraperitoneally 5 times with one week between injections. The mice were sacrificed by heart puncture 10 days after the last injection.

4. *Neutralization (N) test*

N antibody titers were determined by 50% focus reduction tests, using the peroxidase-anti-peroxidase (PAP) staining technique, which was established in our Department (Okuno et al., 1977; Okuno et al., 1978).

5. *Complement fixation (CF) test*

CF tests were performed by the microtiter method (Sever, 1962). Most antigens for the tests were prepared from infected SMB by sucrose-acetone extraction, but in some tests, the infected culture fluids of C6/36 cells were used as CF antigens without any treatment. C6/36 cells grown in 2-ounce bottles were infected with the isolated strains. When CPE appeared in more than 80% of the cell sheet, the culture fluid was harvested and stored in ampoules at −70°C as CF antigen.

6. *Hemagglutination inhibition (HI) test*

Serum specimens for tests were treated with kaolin (acid-washed American standard, Fisher Scientific Co., N.J., USA) and were absorbed with goose red blood cells. HI tests were performed by the method of Clarke and Casals (1958) adapted to a microtiter scale (Sever, 1962).

RESULTS

1. *Virus isolation from acute phase sera of DHF patients*

Table 1 shows the results of serological tests on the sera of patients and of virus isolation.

TABLE 1. *Patients' sera used for virus isolation with C6/36 cells*

Serum No.		Age (yr)	Sex	Date of sampling	HI-titer DEN-1	DEN-2	DEN-3	DEN-4	Immune response	Virus isolation
124	A[a]	11	M	June 16	<20	<20	40	20	Secondary	+
	C[b]			June 29	5120	≥10240	≥10240	≥10240		
126	A	8	F	June 14	<20	<20	<20	<20	Primary	−
	C			June 18	20	80	160	80		
152	A	13	F	June 23	<20	80	<20	20	Secondary	−
	C			June 27	2560	≥10240	2560	≥10240		
176	A	6/12	M	July 2	<20	<20	<20	<20	Primary	+
	C			July 7	40	320	40	80		
177	A	2	M	June 30	<20	<20	<20	<20	Secondary	+
	C			July 7	5120	≥10240	≥10240	≥10240		
SI-8	A	5	F	June 21	40	20	80	80	Secondary	+
	C			June 29	≥10240	5120	≥10240	≥10240		

[a] A: Acute phase serum.
[b] C: Convalescent phase serum.

On the initial inoculations, the C6/36 cells inoculated with acute phase serum of patient SI-8 showed CPE 6 days after inoculation, but the cells inoculated with Chanthaburi specimens did not. In the second blind passages, three of five specimens developed CPE 5–6 days after inoculation. The other two specimens (No. 126 and 152) did not develop CPE, even after the third blind passage.

The immune responses of the paired sera, shown in Table 1, were classified as primary and secondary from the results of HI tests, according to the criteria of Nimmannitya et al. (1969).

As described in the Materials and Methods, specimens from Chanthaburi were kept at rather high temperature for rather long periods and were transported to Bangkok on wet ice. This may be the reason why no virus was isolated from specimens No. 126 and 152. Other possible reasons are discussed later.

TABLE 2. *Results of neutralization tests with isolates*

Isolate	Standard antisera			
	DEN-1	DEN-2	DEN-3	DEN-4
124: SA[a]-4	<20	<20	<20	<20
124: SA-4: SMB[b]-4	<20	60	<20	160
124: SA-4: SMB-10	<20	<20	40	40
176: SA-4	<20	<20	<20	<20
176: SA-4: SMB-4	<20	60	<20	<20
176: SA-4: SMB-10	<20	230	30	<20
177: SA-4	<20	<20	<20	<20
177: SA-4: SMB-5	<20	130	<20	<20
SI-8: SA-4	<20	<20	<20	<20
SI-8: SA-4: SMB-4	<20	200	<20	<20
Homologous viruses	780	1600	1500	880

[a] SA-: C6/36 cell passage.

[b] SMB-: Suckling mouse brain passage.

2. *Identification of isolates by the N test*

The infectivity of isolates grown in C6/36 cells could be assayed by the PAP-staining technique, using BHK21 cells. For identification and typing of the isolates, N tests were performed against standard anti-DEN 1–4 rabbit sera. As shown in Table 2, none of the isolates passaged 4 times in C6/36 cells were neutralized appreciably by any of the standard rabbit sera. Therefore, we tried to adapt the newly isolated strains to SMB by intracerebral inoculation. In the initial passage to SMB, some strains caused no symptoms in suckling mice for more than 3 weeks and some caused slight symptoms in a few of the litter 13–15 days after injections. Strains that failed to cause symptoms in newborn mice were passaged blindly or injected repeatedly with mosquito cell-propagated viruses until inoculated mice showed apparent symptoms. At the 4th passage in SMB, the incubation periods in all strains had become shortened to 6–7 days and all the suckling mice inoculated developed neurological symptoms. At the 10th passage, the incubation periods were further shortened to 4–6 days.

N tests were performed again, using SMB passaged strains of the isolates, and the results are shown in Table 2. Strains No. 176, 177 and SI-8 showed type-specific patterns in N tests against standard anti-DEN 2 rabbit serum after the 4th or 10th passage in SMB. The results in Table 2 indicate that strains No. 176, 177 and SI-8 belong to serotype 2 of DEN virus.

3. *Identification of isolates by CF tests*

As strain No. 124 at a low level of adaptation to SMB could not be identified by N tests, we next performed CF tests. CF antigen of strain No. 124, passaged 4 times in the cells and then 5 times in SMB (124: SA-4: SMB-5) was prepared by sucrose-acetone extraction, and anti-124: SA-4: SMB-5 serum was prepared in adult mice as described in the Materials and Methods.

Figure 1 shows the results of CF tests. An-

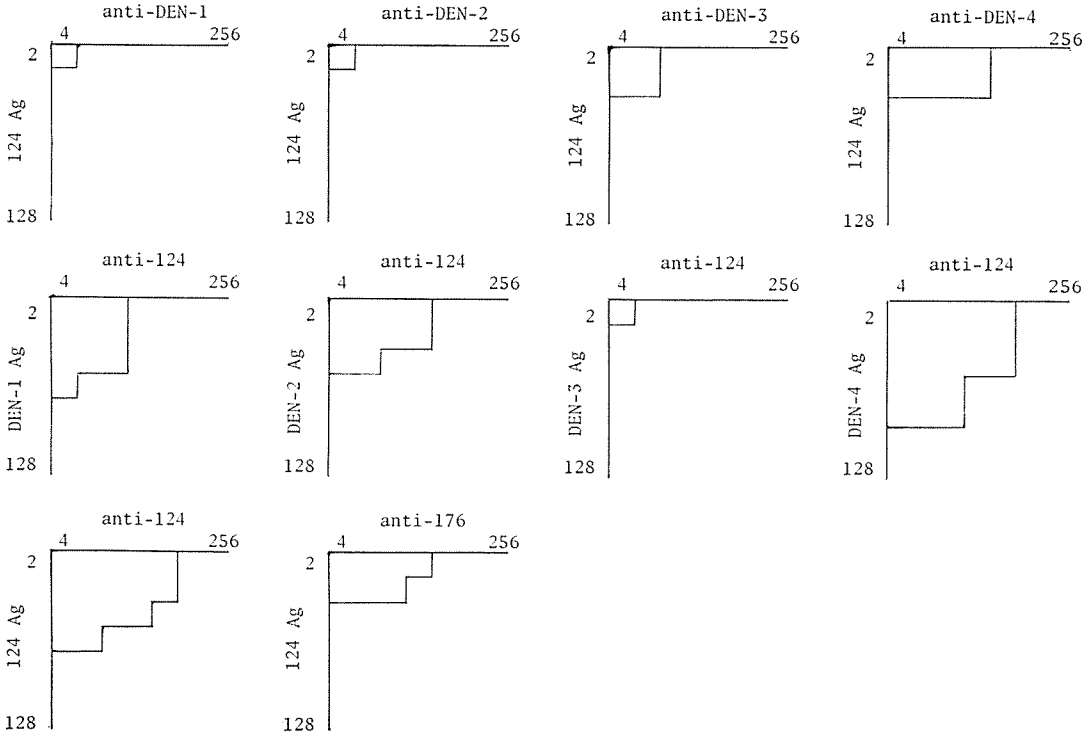

FIGURE 1. CF test on 124: SA-4: SMB-5.

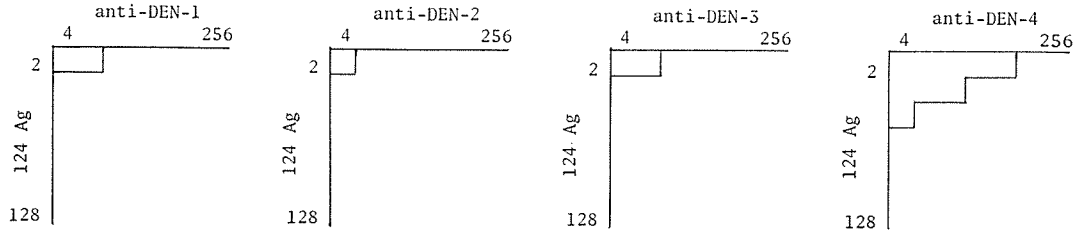

FIGURE 2. CF test on 124: SA-4: SMB-10.

tigen of strain 124: SA-4: SMB-5 showed the strongest reaction with anti-DEN 4 among the mouse sera against the 4 types of DEN virus. Anti-124: SA-4: SMB-5 mouse serum also showed the strongest reaction with DEN 4 antigen among the 4 serotypes of standard antigens. Thus, the No. 124 strain was identified as DEN 4 serotype.

We confirmed results of the CF test using antigen of No. 124: SA-4: SMB-10, which did not react in the N test against anti-DEN sera (Table 2). Figure 2 shows the results, indicating the same conclusion as the results in Fig. 1.

Strains No. 176, 177 and SI-8 were also examined by CF tests to confirm the results of the N tests shown in Table 2. Figure 3 shows the results of CF tests using 176: SA-4: SMB-5 antigen against the 4 serotypes of anti-DEN sera and also using anti-176: SA-4: SMB-5 mouse serum against the 4 serotypes of DEN antigens. Strain No. 176 was iden-

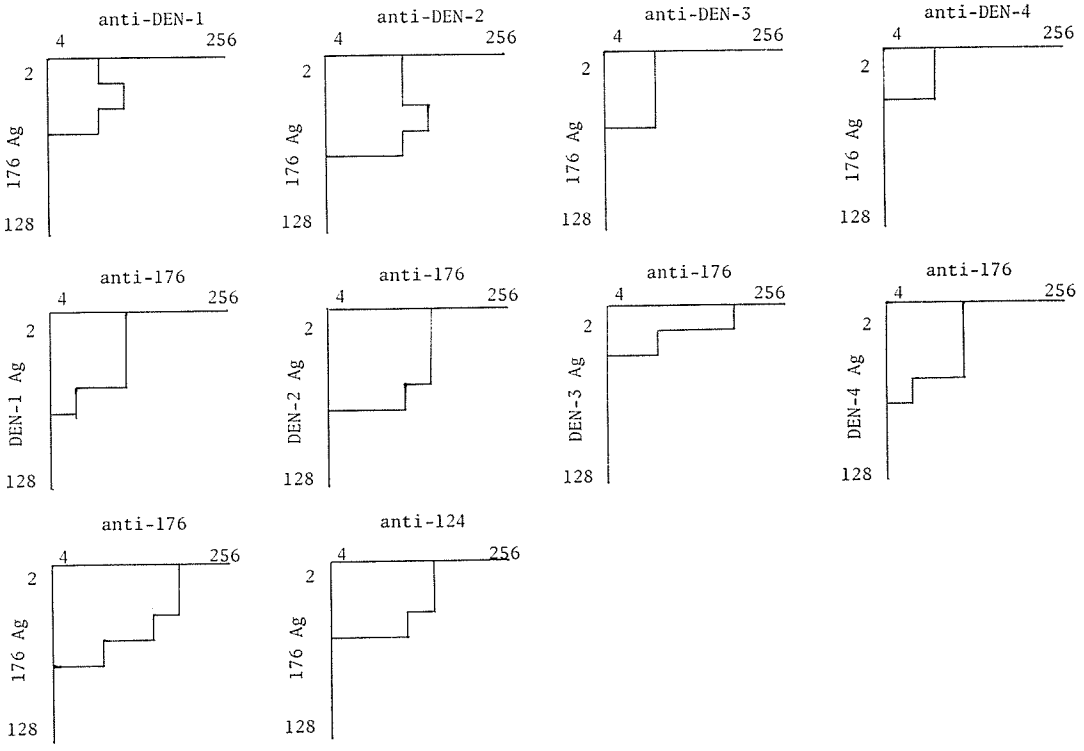

FIGURE 3. CF test on 176: SA-4: SMB-5.

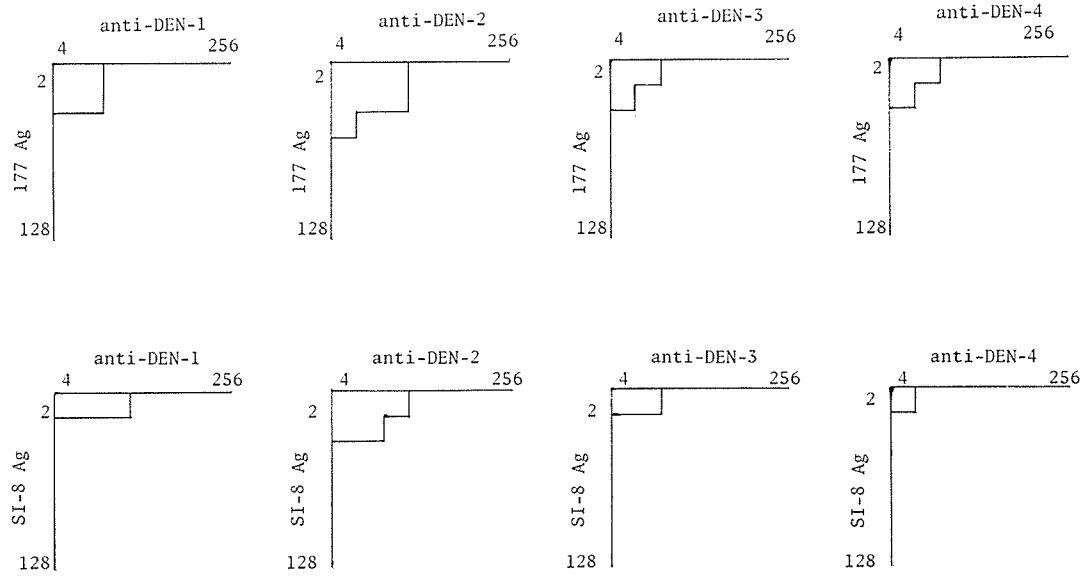

FIGURE 4. CF tests on 177: SA-4: SMB-5 and SI-8: SA-4: SMB-5.

tified as DEN 2 serotype from the patterns of CF tests, which coincided with the results of the N tests shown in Table 2.

Figure 4 shows the results of CF tests, using antigens of 177: SA-4: SMB-5 and SI-8: SA-4: SMB-5 against anti-DEN mouse sera. Both antigens showed the strongest reactions against anti-DEN 2 in the CF tests, which was again consistent with the results of the N tests.

It is too impractical and time consuming to have to prepare CF antigens from infected SMB for identification whenever DEN viruses are isolated using C6/36 cells, especially when the number of isolates is large. Therefore, we tested whether infected culture fluids of C6/36 cells could be used as CF antigens without any concentration or extraction procedures. Figure 5 shows the results. Although the titers of CF antigens in the infected culture fluids of the cells appeared to be lower than those of antigens prepared from infected SMB, the patterns in Fig. 5 indicated that No. 124, 176 and 177 were serotypes DEN 4, DEN 2 and DEN 2, respectively. These results agree well with the results obtained with CF antigens derived from infected SMB. Use of infected C6/36 cell culture fluids as CF antigens makes identification of isolates much easier and faster.

4. *N tests on sera from which viruses were isolated*

Table 3a shows the results of N tests on sera against prototype viruses of the 4 DEN serotypes and also against the homologous isolates.

The types of immune response were more clearly demonstrated by the N test than by the HI test (Table 1). Patient No. 176 had HI antibodies against all of the 4 serotypes, and his convalescent serum had N antibody against the DEN 2 serotype only, clearly indicating that he had been infected with DEN 2 sero-

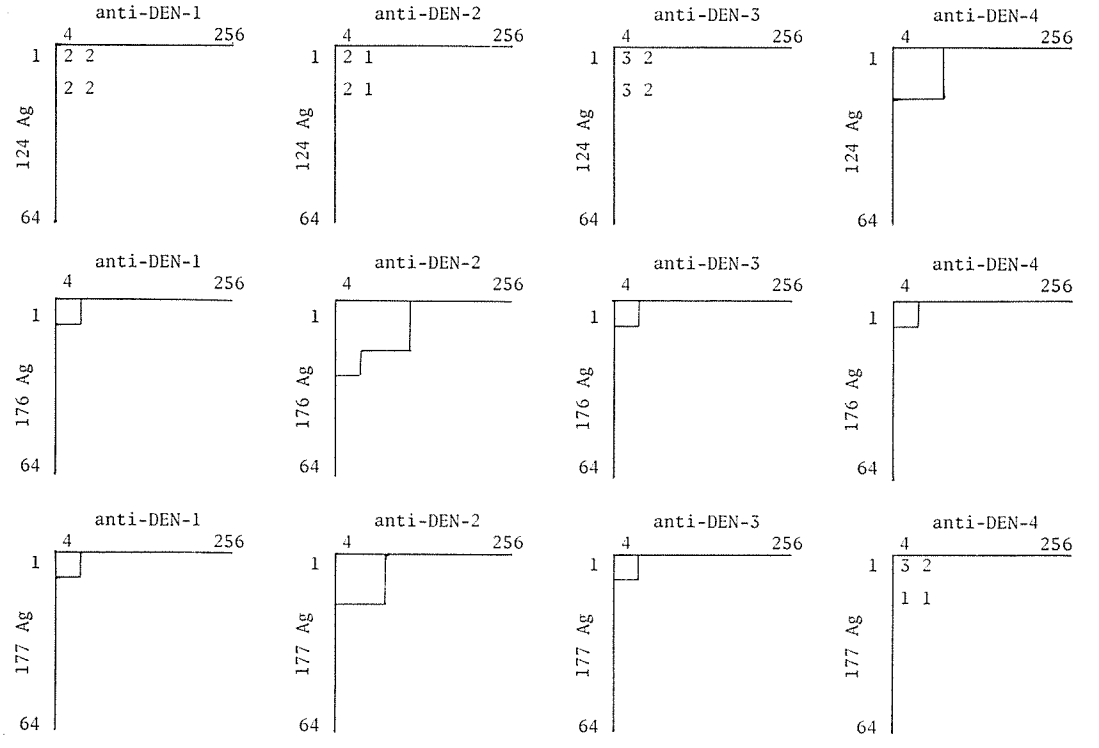

FIGURE 5. CF-test using infected C6/36 cell culture fluids as antigens.

TABLE 3. *Neutralization tests on sera from which viruses were isolated*

(a) Patient sera		Standard viruses				Homologous viruses	Immune response
		DEN-1	DEN-2	DEN-3	DEN-4		
124	A[a]	<20	<20	200	<20	<20	Secondary
	C[b]	4700	2800	15000	5900	500	
176	A	<20	<20	<20	<20	<20	Primary
	C	<20	400	<20	<20	530	
177	A	30	90	220	80	30	Secondary
	C	2100	2600	12000	700	930	

(b)		176 : SA-4 : SMB-4 (DEN-2)	177 : SA-4 : SMB-5 (DEN-2)
176	A[a]	<20	<20
	C[b]	530	840
177	A	40	30
	C	1240	930

[a] A : Acute phase serum.
[b] C : Convalescent phase serum.

type virus only.

It is clear that patient No. 124 had been previously infected with DEN 3 serotype virus because his acute phase serum had N antibody against the DEN 3 serotype only. He was then secondarily infected with DEN 4 serotype virus, as demonstrated by virus isolation and identification as described above. It should be noted that the convalescent phase serum of patient No. 124 gave the highest N antibody titer against the DEN 3 serotype (1: 15,000), with which he had been primarily infected, while the titer against the newly infecting serotype (DEN 4) was not so high (1: 5,900).

Primary infection of case No. 177 may have been possibly caused by the DEN 3 serotype, and the recent infection by the DEN 2 serotype, which was demonstrated by virus isolation and identification.

The convalescent phase serum of case No. 124 gave an N titer of 1: 5,900 against the prototype virus of DEN 4, whereas its titer against No. 124 strain (124: SA-4: SMB-5), which belongs to the DEN 4 serotype as demonstrated by CF tests, appeared to be much lower (1: 500). A similar phenomenon was observed in another case of secondary infection (No. 177). The strain isolated from No. 177 belonged to the DEN 2 serotype and the N titer against the DEN 2 prototype was 1: 2,600, whereas the titer of convalescent phase serum against the homologous isolate (177: SA-4: SMB-4) was 1: 930. In a case of primary infection (No. 176), however, the convalescent phase serum gave an N titer of 1: 530 against the homologous isolate (176: SA-4: SMB-4), which was not lower than that (1: 400) against prototype virus of the DEN 2 serotype.

Table 3b shows the results of N tests on the sera of patients No. 176 and 177 against homologous and heterologous isolates both belonging to the DEN 2 serotype. These tests were performed to know whether isolates of the same serotype would behave similarly in N tests on sera. As shown in Table 3b, the N titers of the two sera against the homologous and heterologous isolates were not significantly different, but too few cases were examined to provide conclusive evidence on this matter.

DISCUSSION

Various kinds of hosts have been used for isolation of DEN viruses from patients with DEN fever or DHF and from field mosquitoes. These hosts include newborn mice (Hammon

et al., 1960; Russell et al., 1968; Nimmannitya et al., 1969), mammalian cells (Russell et al., 1968; Nimmannitya et al., 1969), some species of mosquitoes reared in the laboratory (Rosen and Gubler, 1974; Gubler et al., 1979) and mosquito cells (Singh and Paul, 1969; Race et al., 1978; Race et al., 1979). The last two kinds of hosts seem to be better, because field strains of DEN viruses are not usually pathogenic to newborn mice and many not produce CPE or plaques in vertebrate cells in the initial passage (Rosen and Gubler, 1974). When some of us used newborn mice for virus isolation in 1977, the isolates sometimes disappeared during serial passages in SMB and the infectivity titrations of new isolates gave a non-linear dose-response (unpublished data). In addition adaptation of DEN viruses isolated with human volunteers to newborn mice or adult mice is difficult and time-consuming as described in a report of earlier investigations on DEN (Schlesinger and Frankel, 1952).

In 1978, we used C6/36 cells, a clone of Singh's *Aedes albopictus* cells (Singh, 1967), and isolated 4 strains of DEN virus without problems, as mentioned above. Using C6/36 cells, Tesh (1979) observed an interference phenomenon on titration of infectious human sera. As described in the Materials and Methods, acute phase sera of DHF patients were inoculated onto C6/36 cells at a relatively low dilution (2–5 fold). This " autointerference " by high concentrations of viruses, which were probably present, might have been one reason why we failed to recover viruses from the sera of cases No. 126 and 152 (Table 1).

Another possible reason might be concerned with the ability of C6/36 cells to produce CPE, because CPE was regarded as an indicator of virus growth in this study. Tesh (1979) reported that most DEN strains tested failed to produce recognizable CPE in C6/36 cells although they could grow well in these cells. In our later experiments also, some prototype viruses and SMB-adapted isolates did not produce CPE but grew well in the cells. Therefore, we might have recovered viruses from specimens No. 126 and 152 if we had examined the infectivities of the culture fluids of the C6/36 cells, even though these cells did not show CPE.

It is strange that DEN virus strains isolated and passaged in C6/36 cells showed almost no reaction in N tests against standard anti-DEN rabbit sera (Table 2). CF tests showed that the viruses were DEN viruses and three of four strains reacted in N tests against the same antisera after low level adaptation to SMB. From studies on isolation of JE virus from field mosquitoes, Igarashi et al. (1980) reported that isolates in C6/36 cells were not neutralized by mouse and rabbit antisera against standard JE virus as easily as isolates in SMB. In studies on isolation of DEN viruses, Russell et al. (1968) also observed that isolates propagated in mouse brain were more readily neutralized than isolates from the same source propagated in mammalian cells. The results of N tests with mosquito cell-propagated isolates may represent extreme instances of the trend described above.

The fact that these isolates became reactive with antisera after adaptation to SMB may indicate that some mechanisms such as genetic selection(s) and/or host cell-controlled variation(s) are involved in the process of adaptation to SMB. From studies on adaptation of DEN 2 virus to baby and adult mice, it was suggested that the virus arising in early mouse passages consists of two types of particles, one pathogenic for baby mice only, and the other pathogenic for mice of either age (Schlesinger and Frankel, 1952). Experiments with RNA extracted with phenol from mouse brains infected with " high " and " low " passage DEN 2 virus also suggested that change in viral properties responsible for increased virulence in adult mice resides in the viral genome (Schulze, 1963). These results suggest that genetic selection rather than host cell-controlled variation is involved in the process of adaptation of mosquito cell-propagated virus to SMB.

The prototypes of DEN 1 (Hawaiian strain)

and DEN 2 (New Guinea B strain) were isolated by Sabin (1950) from sera of patients with DEN fever by intracutaneous injection into human volunteers and those of DEN 3 (H-87 strain) and DEN 4 (H-241 strain) were isolated by Hammon et al. (1960) by intracerebral injection into newborn mice. These prototypes have been maintained by passages in SMB. Owing to the histories of isolations and passages of the prototype viruses, the question arises of whether these prototype viruses represent the serological and biological characteristics of all DEN virus populations in nature. With regard to serotype differentiation of the isolates, anti-DEN prototype mouse sera could react well in CF tests with isolates propagated in mosquito cells. Furthermore, sera of DHF patients who had been infected with current DEN viruses also reacted well in N tests with the 4 prototype viruses (see accompanying paper). From these results it seems that the prototypes of DEN 1–4 do in fact represent the serological characteristics of most, if not all, DEN viruses in nature.

On the other hand, two kinds of differences in serologic behaviors between the prototypes and newly isolated viruses were demonstrated in this work. One was in N tests with isolates propagated in C6/36 cells against anti-DEN sera and the other was in N tests with the sera from which the viruses were isolated. In the latter case, the convalescent phase sera of patients with secondary DHF infection neutralized prototype viruses better than isolates derived from the same patients. The N titers against the homologous isolates seemed to remain at a low level of only a type-specific antibody response, while those against prototypes seemed to reach a higher level of type-specific plus subgroup-specific antibody responses (see Discussion of accompanying paper). This is strange but interesting. One possible explanation, pertinent to the pathogenesis of DHF, may be as follows: in secondary DHF infection, the patient produces both type-specific and subgroup-specific N antibodies, but for some reason the latter is not effective against the newly infecting virus, resulting in increased growth of the virus and increased formation of virus-antibody complexes, where the virus is not inactivated appreciably.

We are trying to examine whether this phenomenon is observed in all patients with secondary DHF infection.

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