BIKEN JOURNAL Vol. 25 163–170, 1982

SEROLOGICAL STUDIES ON A CASE OF LABORATORY DENGUE INFECTION[1]

YOSHINOBU OKUNO, TOSHIHIKO FUKUNAGA, MASAYUKI TADANO and KONOSUKE FUKAI

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565, Japan

(Received August 17, 1982)

SUMMARY One of the authors (Y.O.), who had previously been immunized with Japanese encephalitis (JE) vaccine, showed symptoms of typical dengue fever 6 days after accidental infection with a newly isolated dengue type 4 virus strain from a patient with dengue hemorrhagic fever (DHF) in Thailand. His sera were examined by hemagglutination inhibition (HI), complement fixation (CF) and neutralization (N) tests. The JE N antibody titers of his sera were high even on the first day of the illness and remained almost constant during the next year.

Antibodies that reacted with dengue viruses were detected from a very early stage of the illness by all three serological tests. In addition, his convalescent phase sera showed high titers against all 4 types of dengue virus. These data suggest that the dengue infection caused secondary stimulation of antigens of flavivirus.

Sedimentation analysis of antibodies in Y.O.'s serum (day 9) was carried out and IgM antibody that reacted only with dengue type 4 virus and homologous infecting virus was separated. These findings clearly demonstrated that the laboratory infection of Y.O. was primary dengue infection with dengue type 4 virus.

INTRODUCTION

We were interested in a dengue virus strain isolated from a patient (No. 124) with dengue hemorrhagic fever (DHF) in Thailand (Fukunaga et al., 1980), because it was demonstrated by serological tests and also by virus isolation and identification that this patient had been infected primarily with dengue virus type 3 and secondarily with type 4 (Okuno et al., 1980). As far as we know, this is the first DHF patient in whom both primarily and secondarily infecting dengue virus types have been demonstrated. The isolated virus was identified as dengue type 4 virus by the CF test, but not by the N test, which is considered to be the most type-specific of all serological reactions. Therefore, the newly isolated virus was studied in more detail by plaquing on C6/36 cells (Igarashi, 1978), a clone of Singh's

1 A part of this work was presented at the 29th Annual Meeting of the Society of Japanese Virologists held in Tokyo in October 1981.

Aedes albopictus cells (Singh, 1967). During the experiment one of the authors (Y.O.) was accidentally infected with the virus. As it is very difficult to obtain sequential blood samples from patients with natural dengue infection, the infection of Y.O. at this time seemed to provide a good opportunity for following antibody changes over a long period. This paper describes serological studies on Y.O.'s sera, which were collected periodically for one year after the onset of illness.

MATERIALS AND METHODS

1. *Laboratory dengue infection*

One of the authors (Y.O.) was accidentally infected with dengue in the laboratory on May 2, 1980. Details of the origin of the infecting virus were described previously (Fukunaga et al., 1982). Y.O. manifested clinical symptoms first on the night of May 8 and blood samples were collected from him periodically for one year starting after the onset of illness. In our study May 9, 1980 was designated as the first day of the illness. Sera were separated from clotted blood and stored at −20 C for serological tests.

2. *Virus isolation*

Virus was isolated from Y.O.'s serum obtained on the first day of illness using both C6/36 cells (Igarashi, 1978) and suckling mouse brains (SMB). The virus isolated in C6/36 cells is named SI-Y.O.: SA-1 and that isolated in SMB is named SI-Y.O.: SMB-1 in this paper. SI-Y.O.: SMB-7 that had been passaged 7 times in SMB was used for HA and CF antigens.

3. *Prototype dengue and Japanese encephalitis (JE) viruses*

Prototype dengue viruses, type 1 (D1) Hawaiian strain, type 2 (D2) New Guinea B strain, type 3 (D3) H-87 strain and type 4 (D4) H-241 strain, and the prototype JE virus, Nakayama strain, were used. Stock viruses were prepared from 10% homogenates of infected SMB in Eagle's minimal essential medium (MEM). The homogenates were clarified by centrifugation at 10,000 rpm for 30 min and the supernatants were divided into portions and stored in sealed ampoules at −70 C.

For neutralization tests, these prototype dengue viruses were serially passaged 10 times in C6/36 cells and then named D1(SA-10), D2(SA-10), D3(SA-10) and D4(SA-10), respectively.

4. *Neutralization (N) test*

Before the test, sera were heat-inactivated at 56 C for 30 min. The N antibody titers of test sera were determined by the focus reduction method using the peroxidase-anti-peroxidase (PAP) staining technique (Okuno et al., 1977; Okuno et al., 1978). Titers were expressed as values for 50% focus reduction (FR_{50}).

5. *Hemagglutination inhibition (HI) test*

HI tests were performed by the method of Clarke and Casals (1958) as modified for a microsystem (Sever, 1962), using 8 units of hemagglutinating antigens.

6. *Complement fixation (CF) test*

CF tests were performed using 4 CF units of antigens and 2 units of complement of fresh guinea pig sera.

7. *Serum fractionation by sucrose density gradient centrifugation (S-DGC)*

Antibodies in Y.O.'s serum were separated by S-DGC by the method of Kimoto et al. (1970). A sample of 0.1 ml of Y.O.'s serum (9th day) was diluted with 0.4 ml of PBS(−) and the 0.5 ml of diluted sample was layered on 10.5 ml of a 10–40% W/W sucrose gradient and centrifuged at 30,000 rpm for 18 h in a Beckman SW 41 rotor. Twenty fractions were collected dropwise by piercing the bottom of the tube. Two peaks were demonstrated by the HI test. A 0.1 ml sample of each fraction was treated with 7 μl of 2-mercaptoethanol (2-ME) and incubated for 30 min at 37 C. Antibodies in 2-ME treated and untreated fractions were measured by the HI and N tests.

RESULTS

1. *Clinical history*

The onset of illness was sudden with severe chill and malaise. Immediately after these symptoms, fever developed and continued for 6 days. Figure 1 shows the change in body temperature of Y.O.. The pattern of the fever

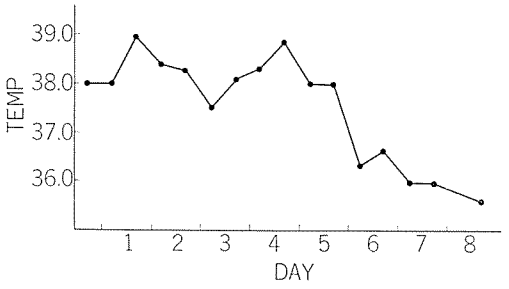

FIGURE 1. Body temperature of Y. O.

was so called "saddleback" which is typical of dengue fever. The fever was associated with headache, myalgia and joint pain and anorexia persisted until day 5 of illness. Diarrhea occurred several times during the febrile period. The inguinal, axillary and cervical lymphnodes were slightly enlarged and tender from day 6 of illness. Soon after the onset of illness, Y.O.'s face became reddish and miliary-size erythema was observed on his neck and chest. On day 6 of illness, a measles-like maculopapular rash appeared on his trunk and extremities, but it disappeared again on the same day leaving slight itching.

Recovery from illness was prolonged because of pronounced asthenia, and depression persisted for several weeks.

Viremia was detected for the first 3 days of the illness, the virus titers were 4.3×10^3 FFU/ml (day 1), 5.3×10^3 FFU/ml (day 2) and 3.0×10^2 FFU/ml (day 3).

2. *N antibody titers of Y.O.'s sera against JE virus*

As Y.O. had been given JE vaccine several times in childhood and also 3 years previously, his N antibody titer against JE virus, Nakayama strain, were investigated. Figure 2 shows the change in his N titer against JE virus for one year after onset of the illness. The N antibody level was high from the first day of the illness and remained at almost this level throughout the next year after a slight increase on day 9. These data indicate that primary dengue virus infection scarcely influenced the JE N antibody titer.

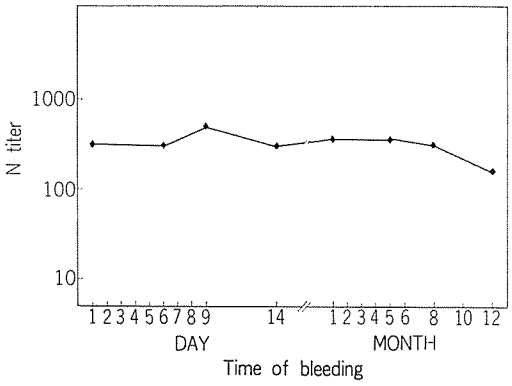

FIGURE 2. N antibody titers of Y. O.'s sera against *JEV* (*Nakayama strain*)

3. *HI and CF antibody titers of Y.O.'s sera*

The HI and CF antibody titers of Y.O.'s sera against prototype D1-4, JE and SI-Y.O.: SMB-7 virus antigens were measured and are shown in Tables 1 and 2, respectively. Both

TABLE 1. *HI antibody titers of Y.O.'s sera*

Time after onset of illness		Virus					
		D1	D2	D3	D4	SI-Y.O.: SMB-7	JE
Day	1	<20	<20	<20	<20	<20	<20
	2	<20	<20	<20	<20	<20	<20
	3	<20	<20	<20	<20	<20	<20
	4	<20	<20	<20	<20	<20	<20
	5	20	20	20	20	40	40
	6	80	80	40	80	160	80
	7	320	160	80	160	320	160
	8	320	160	160	320	640	320
	9	640	320	320	640	1280	320
	14	640	320	160	320	640	320
Month	1	640	160	160	160	320	640
	2	160	160	80	160	160	640
	3	40	80	40	80	160	320
	4	40	80	40	80	80	320
	5	40	40	40	40	80	160
	6	40	40	20	40	80	160
	8	20	20	20	40	40	80
	10	20	20	<20	40	40	80
	12	20	<20	<20	20	20	40

TABLE 2. *CF antibody titers of Y.O.'s sera*

Time after onset of illness		Virus					
		D1	D2	D3	D4	SI-Y.O.: SMB-7	JE
Day	1	<4	<4	<4	<4	<4	<4
	2	<4	<4	<4	<4	<4	<4
	3	<4	<4	<4	<4	<4	<4
	4	<4	<4	<4	<4	<4	<4
	5	4	8	8	16	8	4
	6	8	16	16	128	64	16
	7	16	32	32	128	128	64
	8	32	32	64	256	128	128
	9	64	32	128	512	256	64
	14	32	32	128	256	256	64
Month	1	32	32	64	256	256	64
	2	16	16	32	256	128	16
	3	8	8	16	128	64	8
	4	8	8	16	64	64	8
	5	4	<4	8	32	32	8
	6	4	<4	8	16	16	8
	8	<4	<4	4	16	16	4
	10	<4	<4	<4	16	8	4
	12	<4	<4	<4	16	8	4

HI and CF antibodies were detected from day 5 of illness, reached maxima on day 9 and then decreased gradually. In the HI test, the differences in the antibody levels for 6 virus antigens was too small to indicate the type of infecting virus. However, in the CF test, the antibody titers against D4 and SI-Y.O.: SMB-7 virus antigens were higher than those against other virus types, suggesting that the infecting virus was D4.

The relatively high antibody titers and strong cross reactions among the 6 virus antigens observed in the HI and CF tests may be due to a secondary antibody response in the flavivirus group.

4. *N antibody titers of Y.O.'s sera against "infecting" and "isolated" dengue viruses*

Figure 3 shows the N antibody titers of Y.O.'s sera against infecting (C15/124: SA-9)

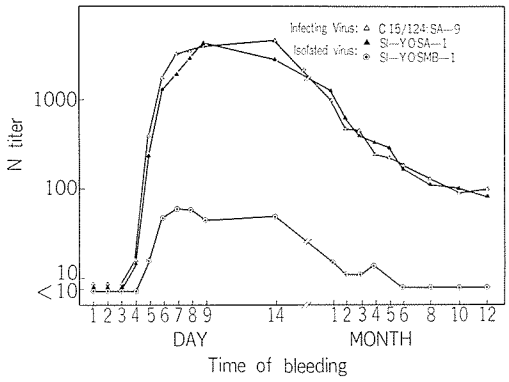

FIGURE 3. N antibody titers of Y. O.'s sera against "*infecting*" and "*isolated*" DEN viruses

and isolated (SI-Y.O.: SA-1 and SI-Y.O.: SMB-1) viruses. The antibody titers against infecting virus and virus isolated from C6/36 cells (SI-Y.O.: SA-1) were detected from day 4 of illness and they increased rapidly to maxima of about 1: 3,000–1: 4,000 after day 7. Then the titers decreased gradually to constant values of about 1: 100 one year after the onset of illness. These two curves of N titers were parallel, suggesting that the properties of the infecting virus did not change during multiplication in Y.O.. On the other hand, when virus isolated in SMB (SI-Y.O.: SMB-1) was used for the N test, the N titer was much lower than those with the other two viruses. The N titer was less than 1: 100 even at its maximum, and it could not be detected (<1: 10) from 6 months after the illness.

This strange phenomenon in the N test was discussed in the preceding paper (Fukunaga et al., 1982).

5. *N antibody titers of Y.O.'s sera against prototype dengue viruses*

Figure 4 shows the N antibody titers of Y.O.'s sera against SMB-passaged prototype dengue viruses. Before the test we expected the sera to neutralize dengue viruses type-specifically, because sera from a DHF patient with primary dengue infection neutralized only

one of the 4 types of dengue virus (Okuno et al., 1980). However, the sera neutralized all dengue virus types to similar extents. The N titers decreased to undetectable levels one year after the illness. As these results were inexplicable, we next performed an N test using prototype dengue viruses that had been passaged 10 times in C6/36 cells (SA). The results in Fig. 5 show that as a whole, the N titers against SA-passaged prototype dengue viruses were higher than those against SMB-passaged prototype dengue viruses. However, the differences between the N titers obtained against SMB-passaged prototype dengue viruses and those against SA-passaged dengue viruses differed considerably for each dengue type (Fig. 4, 5). The biggest difference in N titers was observed with D4, followed by D3 and then D1 and D2. Thus the N titer against D4 was significantly higher than those against other dengue virus types when SA-passaged prototype dengue viruses were used (Fig. 5). Moreover, even one year after illness, the N titer against D4 showed a high and type-specific pattern. The curve of N titers against D4 coincided well with those against the infecting and the SA-isolated viruses shown in Fig. 3. These results also indicated that the infecting virus was D4.

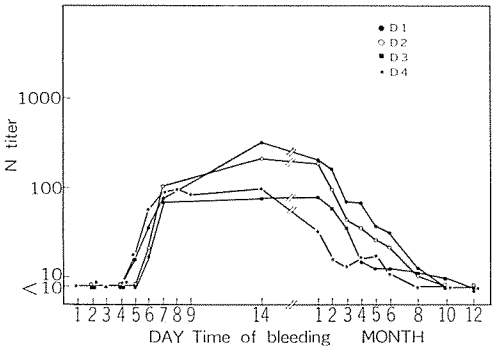

FIGURE 4. N antibody titers of Y. O.'s sera against "*SMB-passaged*" *prototype* DEN viruses

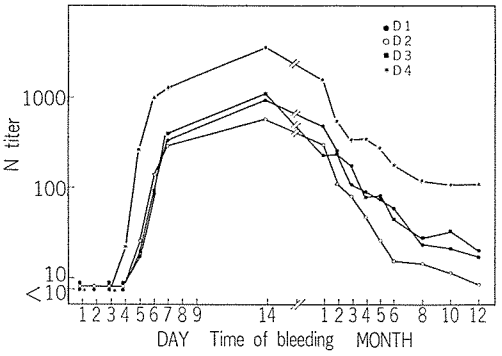

FIGURE 5. N antibody titers of Y. O.'s sera against "*SA-passaged*" *prototype* DEN viruses

6. *Separation of antibody classes in Y.O.'s serum by sucrose density gradient centrifugation (S-DGC)*

Antibodies in Y.O.'s serum on day 9 were analysed by S-DGC. Figure 6 shows the relation between the fraction number and HI antibody titer to SI-Y.O.: SMB-7 virus. Two peaks were separated. Two fractions of each peak were analysed by the HI test using prototype D1-4, JE virus antigens and SI-Y.O.: SMB-7 antigen, and the results are shown in Table 3. The first peak (fraction No. 11, 12) reacted only against D4 and SI-Y.O.: SMB-7 antigens, whereas the second peak (fraction No. 16, 17) reacted equally with all the virus antigens. The antibodies in these fractions with or without 2-ME treatment were studied by the HI test. As shown in Table 4, the first peak was demonstrated to be sensitive, and second resistant to 2-ME. These results shown in Tables 3 and 4 indicate

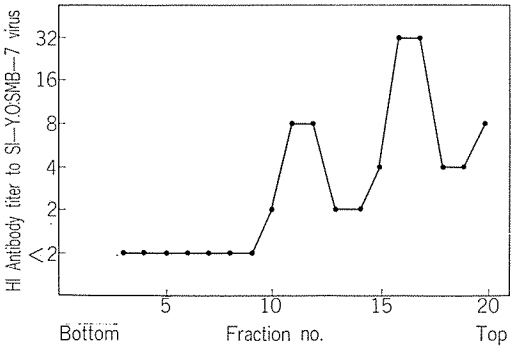

FIGURE 6. Sucrose D.G.C. of Y. O.'s serum (9th day)

TABLE 3. *HI antibody titers of fractions*

Fraction no.	Virus					
	D1	D2	D3	D4	SI-Y.O.: SMB-7	JE
11	<2	<2	<2	8	8	<2
12	<2	<2	<2	8	8	<2
16	16	16	16	16	32	32
17	32	16	16	32	32	32

TABLE 4. *HI antibody titers after 2-ME treatment*

Fraction no.	Virus			
	D4		SI-Y.O.: SMB-7	
	−2ME	+2ME	−2ME	+2ME
11	16	<2	8	<2
12	8	<2	8	<2
16	32	32	32	16
17	32	32	16	16

TABLE 5. *N antibody titers of fractions*

Fraction no.	Virus					
	D1	D2	D3	D4	SI-Y.O.: SA-1	JE
11+12	<2	<2	<2	34	43	<2
16+17	33	9	12	34	27	6

that the first two fractions contained IgM antibody which reacted with D4 and SI-Y.O.: SMB-7 virus antigens type-specifically, while the second two fractions contain IgG antibody that reacted broadly with all the virus antigens. Therefore, the data clearly demonstrated that the illness was due to primary dengue infection with D4.

The N antibody titers in these fractions were also investigated, and results are shown in Table 5. For convenience in the experiment, the two fractions of each peak were mixed and N titer of the mixture was determined. The first peak (Fr[11+12]) reacted only with D4 (SA-10) and SI-Y.O.: SA-1 viruses, whereas the second peak (Fr [16+17]) reacted broadly with all the viruses.

DISCUSSION

The virus that infected Y.O. had been isolated from a patient with DHF using C6/36 cells, a clone of a mosquito cell line, and had been passaged only in the same cells. As dengue virus is transmitted by mosquitoes, this accidental laboratory infection by a needle prick seems to be analogous to natural dengue infection. Grossberg et al. (1959) reported a case of laboratory infection with mouse-adapted dengue virus that occurred in a technician previously infected with JE virus. In their study, the incubation period was longer (18 days) than the usual 5 to 8 days characteristic of dengue naturally acquired by a mosquito bite (Schlesinger, 1977) and the route of inoculation was indirect, rather than direct as by a mosquito bite or needle prick into subcutaneous tissues. In the case of Y.O., the incubation period was 6 days and the clinical symptoms were typical of dengue fever.

As Y.O. had received JE vaccine several times before (most recently 3 years previously), his N antibody against JE virus was demonstrated from the first day of the illness (Fig. 2). However, no HI or CF antibody against JE virus could be detected on the first day of the illness (Table 1, 2). These results indicate that the N test is more sensitive than the HI or CF test for detecting JE antibody. In our recent serological study on JE virus, many cases that gave a negative reaction in the HI test showed high N antibody titers (data not shown).

In our previous work, three different antigenic sites on dengue virus were clearly delineated by serological studies on paired sera from DHF patients (Okuno et al., 1980). There are group-specific (common to all flaviviruses), subgroup-specific (common to all 4 types of dengue virus) and type-specific (specific to each of the 4 types of dengue virus)

antigens, and antibodies corresponding to these antigens can be designated as group-specific (gs), subgroup-specific (sub-gs) and type-specific (ts) antibodies. In serological tests, gs antibody shows extensive cross-reaction in the HI and CF tests with all flaviviruses (Sweet and Sabin, 1954; Casals and Brown, 1954) while ts antibody reacts with only one of the 4 types of dengue virus in the N test (Sabin, 1950; Hammon et al., 1960; Russell and Nisalak, 1967). As for sub-gs antibody, De Madrid and Porterfield (1974) demonstrated by the N test that the 4 dengue serotypes constitute one subgroup, showing no overlap with other flaviviruses.

As observed in the HI and CF tests (Table 1, 2), high and strong cross-reaction among dengue and JE viruses seems to be induced by secondary antigenic stimulation of gs antigen because Y.O. was sensitized to it by administration of JE vaccine. However, gs antibody causes little neutralization, as shown in Fig. 2, where no remarkable change of N titer to JE virus is observed. The results were almost identical to those reported by Wisseman et al. (1962), who inoculated 17D yellow fever vaccine into volunteers who had JE antibody. We also reported that there was no difference between the dengue N titers of pre- and post-JE vaccination sera of persons previously infected with dengue viruses (Quina et al., 1978).

In cases of primary dengue infection, the sera of patients with DHF reacted with only one of the 4 serotypes of dengue virus in the N test (Okuno et al., 1980). Although the infection at this time was demonstrated to be primary dengue infection with D4 (Table 3, 4, 5), the sera of Y.O. neutralized all the dengue serotypes (Fig. 4, 5). Similar observations were reported by Schlesinger et al. (1956) and Bancroft et al. (1981). They reported trials of infection with attenuated dengue vaccine of persons who had previously been immunized with yellow fever vaccine and their results showed that the post- vaccination sera neutralized not only the dengue serotype used for vaccination but also other dengue serotypes. The antibody that was responsible for these strong cross-reactions among dengue serotypes in the N test must be sub-gs antibody, because we found previously that the sub-gs antibody detected in the sera of DHF patients with secondary dengue infection was the only possible antibody that could neutralize all the dengue serotypes equally well (Okuno et al., 1980). It is not clear why the N titers against all dengue serotypes rose after infection of one dengue serotype in persons who had been immunized with flaviviruses of other subgroups such as JE or yellow fever virus.

The higher efficiency of neutralization of SA-passaged dengue virus than of SMB-passaged virus is shown in Figs. 3, 4 and 5. For determination of this phenomenon was specific for this case only, it is necessary to continue N tests on dengue immune sera using both SA- and SMB-passaged dengue viruses.

REFERENCES

Bancroft, W. H., Top, F. H. Jr., Eckels, K. H., Anderson, J. H. Jr., McCown, J. M., Russell, P. K. 1981. Dengue-2 vaccine: Virological, immunological, and clinical responses of six yellow fever-immune recipients. Infect. Immun. 31: 698–703.

Casals, J., Brown, L. V. 1954. Hemagglutination with arthropod-borne viruses. J. Exp. Med. 99: 429–449.

Clark, D. H., Casals, J. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. 7: 561–573.

Fukunaga, T., Okuno, Y., Srisupaluck, S., Auwanich, W., Rojanasuphot, S., Sangkawibha, N., Kasemsarn, P., Dharakul, C. 1980. Serological and virological studies on patients with dengue hemorrhagic fever (DHF) in Chanthaburi Pro-

vince, Thailand. II. Serological characteristics of viruses isolated from DHF patients using a clone of Singh's *Aedes albopictus* cells. Biken J. 23: 123–133.

Fukunaga, T., Okuno, Y., Tadano, M., Fukai, K. 1982. Neutralization characteristics of dengue viruses isolated in mosquito cell culture and suckling mouse brain from a case of laboratory dengue infection. Biken J. 25: 139–147.

Grossberg, S. E., Scherer, W. F. 1958. Immunity in group B arthropod-borne virus diseases. Accidental dengue I virus infection in a laboratory worker with antibodies to Japanese encephalitis virus. Am. J. Hyg. 69: 60–67.

Hammon, W. Mcd., Rudnick, A., Sather, G. E. 1960. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science 131: 1102–1103.

Igarashi, A. 1978. Isolation of Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol. 40: 531–544.

Kimoto, T., Fukunaga, T., Isshiki, G., Takeuchi, T., Sato, Y., Kozaki, K., Yoneda, S., Nagase, S. 1970. Case report of congenital rubella in Osaka Prefecture. Biken J. 13: 377–381.

Madrid, A. T. de, Porterfield, J. S. 1974. The flaviviruses (group B arboviruses): A cross-neutralization study. J. Gen. Virol. 23: 91–96.

Okuno, Y., Fukunaga, T., Srisupaluck, S., Kasemsarn, P., Dharakul, C., Sangkawibha, N. 1980. Serological and virological studies on patients with dengue hemorrhagic fever (DHF) in Chanthaburi Province, Thailand. I. Serological studies on paired sera from DHF patients by neutralization (N), hemagglutination inhibition (HI) and staining tests. Biken J. 23: 113–121.

Okuno, Y., Igarashi, A., Fukai, K. 1978. Neutralization tests for dengue and Japanese encephalitis virus by the focus reduction method using peroxidase-anti-peroxidase staining. Biken J. 21: 137–147.

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.

Quina, M. A., Soe Thein, Auwanich, W., Okuno, Y., Igarashi, A., Fukai, K. 1978. Changes in dengue and Japanese encephalitis (JE) antibody after JE vaccination. Biken J. 21: 149–159.

Russell, P. K., Nisalak, A. 1967. Dengue virus identification by the plaque reduction neutralization test. J. Immunol. 99: 291–296.

Sabin, A. B. 1950. The dengue group of viruses and its family relationships. Bacteriol. Rev. 14: 225–232.

Schlesinger, R. W. 1977. Virology Monographs, 16. Dengue viruses. Springer-Verlag, New York.

Schlesinger, R. W., Gordon, I., Frankel, J. W., Winter, J. W., Patterson, P. R., Dorrance, W. R. 1956. Clinical and serologic response of man to immunization with attenuated dengue and yellow fever viruses. J. Immunol. 77: 352–364.

Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88: 320–329.

Singh, K.R.P. 1967. Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr. Sci. 36: 506–508.

Sweet, B. H., Sabin, A. B. 1954. Properties and antigenic relationship of hemagglutinins associated with the dengue viruses. J. Immunol. 73: 363–373.

Wisseman, C. L., Jr., Sweet, B. H., Kitaoka, M., Tamiya, T. Immunological studies with group B arthropod-borne viruses. I. Broadened neutralizing antibody spectrum induced by strain 17D yellow fever vaccine in human subjects previously infected with Japanese encephalitis virus. Am. J. Trop. Med. Hyg. 11: 550–561.