BIKEN JOURNAL Vol. 11, 41–49, 1968

ANTIBODY AGAINST JAPANESE ENCEPHALITIS VIRUS IN SERA OF DENGUE HEMORRHAGIC FEVER PATIENTS IN THAILAND[1]

AKIRA IGARASHI, KONOSUKE FUKAI

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Osaka, JAPAN

SOMPOP AHANDRIK and PRAKORB TUCHINDA

Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, Bangkok, THAILAND

(Received December 23, 1967)

SUMMARY By the hemagglutination-inhibition (HI) test in the diagnosis of dengue hemorrhagic fever, there is a strong correlation between the antibody titers against Japanese encephalitis virus (JEV) and dengue virus types 1–4. In the neutralization test (NT) using a 50% plaque reduction method, the reactivity of the sera was more specific with respect to the causative agents, but there was still a slight but certain increase in NT antibody against JEV in about 90% of the sera of patients with dengue hemorrhagic fever which showed a positive increase in HI titer against dengue viruses. When the JEV antibody titers were plotted against the time after onset of the disease, the NT antibody was found to increase from the fourth day reaching a plateau on the sixth day, with a mean logarithmic value of 1.06. HI antibody continued to increase until the seventh day and had a maximum titer of 1280–2560. Thus after the seventh day of the disease, the logarithmic value of HI/NT was in the order of 2.0 for JEV.

INTRODUCTION

Mosquito-borne hemorrhagic fever has been, and still is a major viral disease of children in Thailand since its explosive outbreak in 1958. Dengue viruses of multiple types are considered to be the principal etiological agents causing severe disease symptoms, although Chikungunya virus is also counted among the etiological agents causing a smaller number of the cases (HALSTEAD *et al.*, 1963, HALSTEAD, 1966). One of the characteristic responses in dengue hemorrhagic fever revealed by serological tests is that the antibody response is high and broad. This phenomenon is referred to as a "secondary type" of antibody reaction to group B arbovirus antigens (HALSTEAD and YAMARAT, 1965, HALSTEAD, 1966) including Japanese encephalitis virus (JEV).

By chance in the Virus Research Institute, Bangkok, a paired sera of a case of dengue

1 This work was perfomed by cooperation between Thailand and Japan under the Colombo plan.

hemorrhagic fever were found to show a certain rise of NT antibody to JEV as tested by the plaque reduction test when treated in parallel with several specimens of sera of patients suspected of encephalitis. Thus it seemed necessary to see whether patients with dengue hemorrhagic fever always acquired some degree of NT antibody to JEV, and, if so, the extent of the heterologous NT antibody titer. The result of this study are reported in this paper.

MATERIALS AND METHODS

1. *Virus*

Dengue viruses, type 1 Hawaiian strain (D1), type 2 TR1751 strain (D2), type 3 H87 strain (D3) and type 4 H241 strain (D4) and Japanese encephalitis virus Nakayama strain (JEV) were used. All the virus strains were propagated in suckling mouse brain.

2. *Hemagglutination and hemagglutination-inhibition (HI)*

These tests were carried out according to the method of CLARKE and CASALS (1958), using antigens extracted from infected suckling mouse brains with acetone-ether and sucrose-acetone method. Two fold serial dilutions of the sera were made on a plastic tray starting from 20 fold serum dilution.

3. *Tissue culture*

Primary cultures of chick embryo cells (CEC) were prepared by the method of PORTERFIELD (1960) and grown in 2 ounce rubber-stoppered prescription bottles with YLE medium (0.1% yeast extract, 0.5% lactalbumin hydrolyzate and 0.55% glucose in Earle's balanced salt solution with antibiotics and phenol red) supplemented with 10% bovine serum. Five ml of CEC suspension containing 1.5×10^7 cells were put into each culture bottle and incubated at 37°C for 24 hours to make cell monolayers.

An established cell line of African green monkey kidney (VERO) was supplied by Dr. Oya's laboratory in the National Institute of Health of Japan, Tokyo, at the 184th passage level and cultivated as described elsewhere (IGARASHI and TUCHINDA, 1967).

4. *Assay of virus infectivity*

Virus infectivities were assayed by the plaque technique. Virus samples were diluted serially 10 fold with 0.75% bovine plasma albumin (BPA) fraction V (Armour) in YLE, pH 7.6, at 4°C, and 0.2 ml of diluted specimen was inoculated onto cell monolayers using CEC for JEV and VERO for dengue virus. Inoculated viruses were allowed to become adsorbed at 37°C for 2 hours, spreading virus solutions over the cell sheets every 30 min. Then they were covered with 5 ml of agar overlay medium per bottle. The composition of the overlay medium for the JEV-CEC system was the same as that described by HASHIMOTO and PRINCE (1963) except that the serum was replaced by BPA at 0.2% final concentration. The first overlay medium for D4 was prepared by mixing 2% melted agar (Difco Special Noble) in distilled water with an equal volume of double strength YLE medium (in this case containing 0.2% glucose) supplemented with 4% bovine serum. After solidification of the agar, bottles were inverted and incubated at 37°C. The plaques of JEV began to appear on the third day and they were counted until the fifth day after inoculation. In the case of D4, after one week's incubation, bottles were covered with one ml of the second overlay medium, which was the same as the first but containing 0.02% neutral red, and the plaques were counted from the 8th until the 10th day after inoculation. Virus titers were calculated to give plaque forming units (PFU) per ml..

5. *Neutralization test (NT) of the serum*

The 50% plaque reduction method for measuring the serum NT titer was performed essentially according to the method of HASHIMOTO and PRINCE (1963), except that sera were serially diluted 5 fold with 0.75% BPA in YLE, starting from the undiluted specimen, Serum NT titers were expressed as the negative decimal logarithm of the dilution of the serum which reduced the plaque number to 50% of that in the control specimen.

6. *Serum specimen*

A number of specimens of human sera from patients clinically diagnosed or suspected of having mosquito-borne hemorrhagic fever had been sent to the Virus Research Institute in Bangkok for laboratory diagnosis. These were tested by the HI and complement-fixation test with D1, D2, D3, D4, and Chikungunya antigens. Sera received from August 1965 until July 1966 were used, and they were also tested by HI with JEV antigen. Of these specimens,

the paired sera that showed a positive rise of HI titer for dengue viruses with little or no detectable HI titer in the acute specimens were selected. They were tentatively considered as sera of dengue hemorrhagic fever. Paired sera which showed negative HI titers for dengue viruses both in acute and convalescent specimens were also selected. These sera were tentatively considered as dengue hemorrhagic fever negative controls. Records were inadequate and the age, sampling time and time of onset of disease of the specimens had not always been recorded. However, patients were not more than 12 years old and the average sampling time with the standard deviation of sera of the acute stage was 3.2 $\pm$1.29 days and that of sera of the convalescent stage was 8.0$\pm$2.09 days after the onset of the disease in 54 pairs of samples from cases of dengue hemorrhagic fever. All the sera were inactivated at 56°C for 30 min before tested for NT antibody.

7. *Statistical method*

Regression lines were obtained by the least square method and the correlation coefficient (r) was calculated by the conventional method. The average titers of antibody were expressed as geometrical mean values. Analysis of variance and Student's t-test were performed by the method described by SNEDECOR (1946).

RESULTS

1. *Correlation between HI titer for dengue viruses and that for JEV*

As expected from the broad reactivity of the sera of cases of dengue hemorrhagic fever, a high correlation between the HI titer for dengue viruses and that for JEV was found in 226 specimens of sera (Fig. 1, No. 1–4). The r-value was especially high for D4-JEV and D2-JEV.

2. *Rise of NT antibody against JEV*

Paired sera of dengue hemorrhagic fever and negative control specimens were tested for NT antibody against JEV by plaque reduction. Results are shown in Figs. 2 and 3. There was some rise of NT antibody against JEV in 80 out of 89 pairs of sera of dengue hemorrhagic fever, and the average titer of difference between the convalescent and the acute specimens was calculated to be 0.610 with the standard deviation of 0.455. But in the negative control specimens only 7 out of 18 pairs showed an increase of NT antibody against JEV, having the average titer of difference between the convalescent and the acute specimens of $-$0.094 with the standard deviation of 0.614. Applying the Student's t-test, the difference between these two groups of specimens was considered to be highly significant ($d.f.=105$, $t=5.499$, $P<0.001$). Thus the null hypothesis that these two groups of samples were selected out of the same population was rejected with the reliability of more than 99.9%.

3. *Rate of appearance of antibody against JEV*

Of the serum samples shown in Fig. 2, 111 specimens with records of sampling time related to the time of onset of the disease were selected. The geometrical mean values of the HI and NT titer for JEV on each day after the onset of the disease were calculated. There were few specimens for the first, 10th, 11th and 12th days (5, 4, 4 and 2 specimens, respectively), so the values for these days may not be very reliable. The result (Fig. 4) shows the rate of appearance of HI and NT antibody against JEV in sera of cases of dengue hemorrhagic fever. The NT titer increased from the 4th until the 6th day of the disease, reaching a plateau value. In 49 specimens taken after the 6th day of the disease, the mean value of the NT titer to JEV was 1.06$\pm$0.52. But the HI titer continued to increase until the 7th day of the illness attaining a titer of 1280–2560. Thus after the 7th day of the disease, the ratio of HI to NT for JEV was in the order of 10^2.

4. *Evidence suggesting that the rise of JEV-NT is not the result of previous JEV-immunity*

The observed rise in the JEV-NT titer in sera of patients with dengue hemorrhagic fever might not be the result of dengue infection itself but rather the result of a previous JEV-

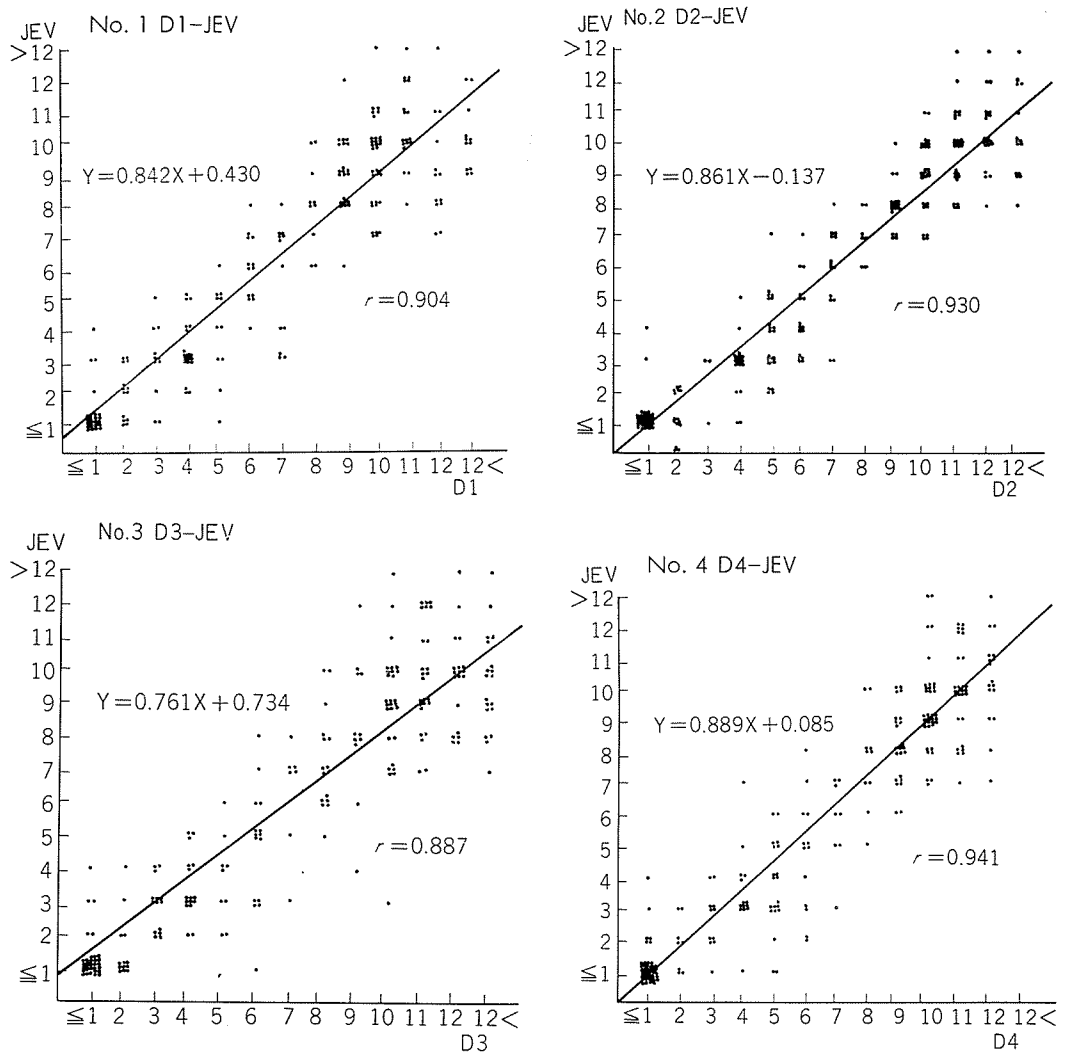

FIGURE 1 Cross reaction between dengue virus and JEV as tested by HI in sera of dengue hemorrhagic fever.
HI titers are expressed as $\log_2(HI/10)$.

infection. To test this possibility, the relation between the preexinsting JEV-NT titer and the extent of rise in the NT titer after dengue infection was first studied. Of the data in Fig. 2, the 26 pairs which had sera taken in an acute stage within 3 days of the onset of the disease and convalescent sera taken after the 6th day of the illness were selected. From the results in Fig. 4, the NT titer for JEV is not considered to increase before the 3rd day, and after the 6th day of the illness it is considered to have reached a plateau. Data on these paired sera are rearranged in Fig. 5, to show the relation between the preexisting NT titer for JEV and the difference between the NT titer in the convalescent and acute stages.

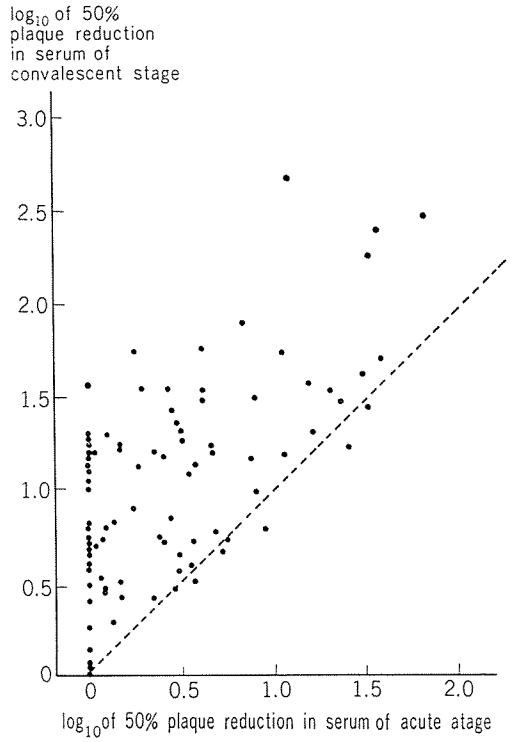

FIGURE 2 Rise of NT antibody against JEV in serum of dengue hemorrhagic fever patient tested by the plaque reduction method.

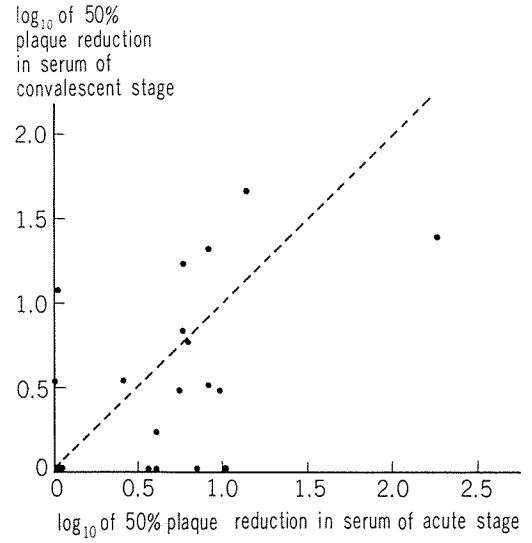

FIGURE 3 NT antibody against JEV in serum with negative dengue antibody tested by the plaque reduction method.

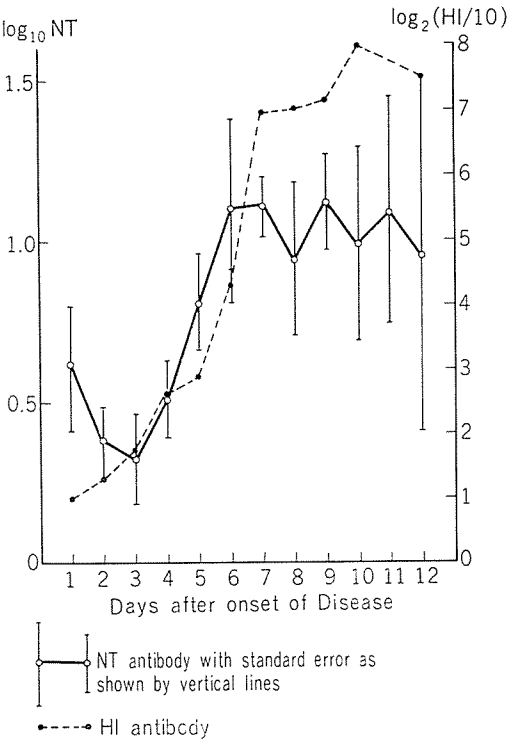

FIGURE 4 Appearance of NT and HI antibody against JEV in serum of dengue hemorrhatic fever patient.

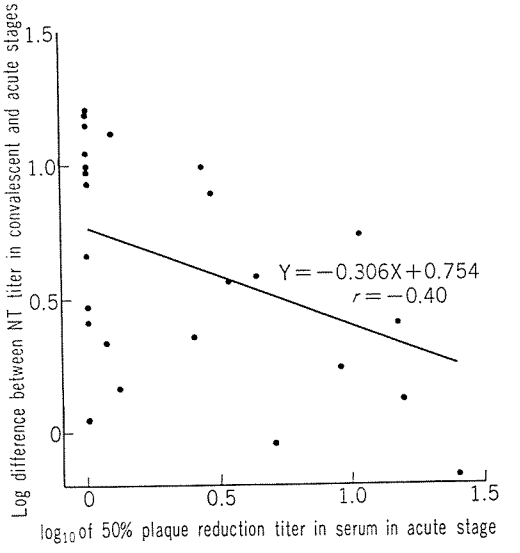

FIGURE 5 Relationship between preexisting NT titer for JEV in serum of acute stage and NT titer rise in serum after dengue hemorrhagic fever.

TABLE 1 *NT titer against JEV in sera of convalescent cases of dengue hemorrhagic fever arranged in five age groups*

	Age group (years)					Total
	<1	1-4	5-6	7-8	9≦	
X	0.59	1.55	0.99	1.32	1.21	
	1.23	1.20	0.28	0.71	0.99	
	0.72	0.59	1.75	1.15	0.25	
	1.42	0.66	1.31	1.09	1.21	
	1.36	0.41	0.46	1.64	0.73	
	0.73	0.04	1.74	2.41	1.54	
	0.79	1.29	1.09	2.69	1.53	
	0.97	1.44	0.63	0.80	1.28	
	0.57	0.66	1.24	0.46	0.03	
	1.54	1.19	0.73	0.71	1.49	
	0.85	0.58	1.04	1.12	1.12	
		1.58	1.23		1.53	
			1.25		1.18	
			1.71			
			0.40			
SX	10.77	11.19	15.85	14.10	14.09	66.00
k	11	12	15	11	13	62
$\bar{x}$	0.979	0.923	1.056	1.281	1.183	1.065
SX^2	11.762	13.218	19.973	23.101	18.063	86.117
$(SX)^2/k$	10.545	10.435	16.748	18.074	15.271	

There is a slight negative correlation between these two variables ($r = -0.40$). There seems to be a slight tendency for a larger difference between the NT titer for JEV in the convalescent and acute stages when the preexisting NT titer for JEV is lower.

Second, data on sera of convalescent cases taken more than 6 days after the onset of the disease which had records of the age of the patient were selected. They were rearranged in 5 age groups (Table 1). The average NT titer for JEV in the convalescent stage was calculated for each group. Groups of older children would have had a greater chance to have had JEV infection before suffering from dengue infection than groups of younger children. The result indicates that the NT titers in older children tended to be a little higher than those in younger children but the difference between the groups was not significant compared with the difference between individuals in a single group. Moreover, the null hypothesis that these 5 groups were selected from the same population could not be rejected by analysis of variance (Table 2).

TABLE 2 *Analysis of variance of the results in Table 1*

Source of variation	d.f.	Sum of squares	Mean square
Between age groups	4	0.815	0.2038
Within age groups	57	15.044	0.2639
Total	61	15.859	0.2600

$F = 1.294$ $P > 0.20$

The null hypothesis cannot be rejscted.

5. *NT titer against dengue virus type* 4

In the data in Fig. 1, there is a higher correlation between the HI titer for JEV and D4

than the HI titer for JEV and other dengue viruses. Accordingly, the NT titer for D4 was assayed on a small number of paired sera to see whether there was some difference in the NT titers for JEV and D4, in contrast to the results of the HI test. Although the number of test specimens was too small, mainly because of the technical difficulty of plaque titration of D4, the results in Table 3 suggest that the reactivity of the sera were more specific in respect to the causative agent when the NT titer was measured, but a conclusive etiological diagnosis and the problem of the relation between the NT titers of these viruses require more extensive tests for NT antibodies to all types of dengue viruses.

TABLE 3 *HI and NT antibody in paired sera of cases of dengue hemorrhagic fever*

Code No. (age)	HI					$\log_{10}$ NT		Date taken	(days after onset)	Complement-fixation tets
	Den-1	Den-2	Den-3	Den-4	JEV	Den-4	JEV			
2042 A	<40	40	<40	<40	40	<0.65	0.40	?		
(?) C	80	40	40	320	160	1.35	1.17			
2064 A	<40	80	<40	<40	—	2.06	0.18	?		
(?) C	80	2560	80	320	320	2.26	0.48			
2067 A	320	320	2560	320	<20	1.42	1.09	?		
(?) C	10240	10240	>20480	>20480	2560	3.10	1.75			
2303 A	80	<40	<40	<40	40	1.00	0.0	Aug. 25 '65	(3)	
(4) C	>40960	>20480	>20480	>20480	>20480	2.05	1.19	Aug. 29 '65	(7)	D1, D3 : 512
2352 A	<40	<40	<40	<40	40	0.65	0.0	Dec. 31 '65	(5)	
(3m) C	10240	<40	80	160	160	1.00	0.721	Jan. 4 '66	(9)	
2382 A	<80	<80	<80	<80	<80	0.65	0.0	Jan. 4 '66	(3)	
(?) C	<80	<80	320	40	640	2.05	0.40	Jan. 10 '66	(9)	
2394 A	<40	<40	<80	<40	<20	0.65	0.0	Jan. 11 '66	(3)	
(5m) C	80	80	80	160	<20	1.35	1.42	Jan. 15 '66	(7)	
2592 A	<40	<40	<40	160	<20	1.76	0.09	Apr. 19 '66	(2)	
(9) C	5120	>20480	10240	>20480	5120	3.46	1.21	Apr. 24 '66	(7)	D3 : 512
2684 A	160	160	160	640	320	0.65	0.0	Jun. 14 '66		
(9) C	>10240	>10240	>20480	>20480	5120	1.35	1.53	Jun. 20 '66		

DISCUSSION

Sera taken from cases of dengue hemorrhagic fever showed a high titer of HI antibody against JEV. There was an especially close relationship between JEV and D4, and JEV and D2. This implies that when a patient suffers from dengue hemorrhagic fever he also acquires a high titer of HI antibody against JEV, although a little lower than for dengue viruses. This result is consistent with the description of the broad reactivity of sera of cases of dengue hemorrhagic fever with group B arbovirus antigens (HAMMON and SATHER, 1964, HALSTEAD and YAMARAT, 1965, HALSTEAD, 1966), and it is also expected from the cross-reactivity of JEV and dengue viruses revealed by the HI test in laboratory experiments (CASALS and BROWN, 1954, CASALS, 1962), but the degree of cross-reaction is much

greater in cases of dengue hemorrhagic fever.

About 90% of the paired sera from cases of dengue hemorrhagic fever were found to show a slight but definite increase in NT titer for JEV, in contrast to the negative controls. Using the method of mouse brain titration, HAMMON and SATHER (1964) reported the presence of NT antibody to JEV in sera of some cases of dengue hemorrhagic fever. They found that the NT titer for JEV is not detectable or not significant in the acute stage within 4 days in cases of primary group B arbovirus infection. Their findings are consistent with the results given in this report. The time course of development of the HI titer for JEV was found to parallel with the result with dengue viruses reported by OKUNO (1966). It would be interesting that the NT titer for JEV begins to increase from the 4th day of the disease, when the disease enters a new stage of abrupt deterioration and shock, and the largest number of deaths have been recorded on the 4th day (HALSTEAD, 1965, 1966). The NT antibody to JEV reaches a plateau after the 6th day of the disease, but at present there is no evidence that this heterologous antibody persists for long. However, in a few cases the NT titer for JEV was demonstrated even 3 weeks after the onset of the disease. Thus, when a patient suffers from dengue hemorrhagic fever, he acquires a certain amount of NT antibody against JEV as well as a high HI titer, at least for a short period of time.

The relation between the extent of rise in NT antibody against JEV and previous JEV infection was studied. No evdience was found that the rise of NT antibody against JEV was the result of an anamnestic reaction to preexisting memory of JEV infection.

More extensive research, using every type of dengue virus, is necessary to obtain information on the prevalence of the viruses and the pattern of response of sera to multiple infection with group B arboviruses. However in a study with a few paired sera the NT seemed to be the more specific reaction with respect to the causative agent.

WISSEMAN *et al.*, (1966) reported that subjects with preexisting JEV antibodies developed a low level of type 1 dengue antibody following vaccination with 17D yellow fever vaccine, but that these subjects developed classical type 1 dengue fever when exposed to unmodified type 1 dengue virus. The protective role of the heterologous NT antibody against JEV found in sera of convalescent cases of dengue hemorrhagic fever is uncertain, but OYA (1965) reported that JEV-NT antibody at a level which was detectable by plaque reduction at 1: 10 serum dilution can inhibit the development of the disease in mice which are inoculated peripherally with 10^4 LD_{50} of JEV. If this amount of peripheral antibody is sufficient to protect against manifestation of the disease in man, and if the NT antibody against JEV observed in this study persists for a long time, it may have some significance in the prevalence and epidemiology of JEV in Thailand, where JEV infection is not considered so grave a problem as dengue hemorrhagic fever although there are many vectors and hosts and some reports of isolation of JEV. It may be that the widespred occurrence of one virus in a certain area may modify the spread and decrease in severity of diseases caused by other related viruses due to serological cross-reactivity (CLARKE and CASALS, 1964).

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Dr. A. Oya of the National Institute of Health of Japan, Tokyo, for supplying the VERO cell strain, and to Miss Suntaree of the Virus Research Institute, Bangkok, for her excellent technical help and for preparation of HI antigens.

REFERENCES

CASALS, J. (1962). Antigenic relationship among arthropod-borne viruses ; Effect on diagnosis and cross immunity. *In* LIBIKOVA, H. [Ed.] Biology of viruses of the tick-borne encephalitis complex. pp

53–66. Academic Press, New York.

CASALS, J. and BROWN, L. V. (1954), Hemagglutination with arthropod-borne viruses. *J. Exptl. Med.* **99**, 429–449.

CLARKE, D. H. and CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**, 561–573.

CLARKE, D. H. and CASALS, J. (1964). Arboviruses ; group B. *In* HORSFALL, F. L. JR. and TAMM, I. [Ed.] Viral and rickettsial infections of man. pp 606–658. Lippincott Co. Philadelphia.

HALSTEAD, S. B. (1965). Dengue and hemorrhagic fevers of Southeast Asia. *Yale J. Biol. Med.* **37**, 434–454.

HALSTEAD, S. B. (1966). Mosquito-borne haemorrhagic fevers of South and Southeast Asia. *Bull. WHO.* **35**, 3–15.

HALSTEAD, S. B. and YAMARAT, C. (1965), Recent epidemics of hemorrhagic fever in Thailand. Observations related to pathogenesis of a " new " dengue disease. *Am. J. Public Health* **55**, 1386–1395.

HALSTEAD, S. B., YAMARAT, C. and SCANLON. J. E. (1963). The Thai hemorrhagic fever epidemic of 1962. A preliminary report. *J. Med. Assoc. Thailand* **46**, 449–461.

HAMMON, W. McD. and SATHER, G. E. (1964). Virological findings in the 1960 hemorrhagic fever epidemic (dengue) in Thailand. *Am. J. Trop. Med. Hyg.* **13**, 629–641.

IGARASHI, A. and TUCHINDA, P. (1967). Studies on Chikungunya virus. I. Plaque titration on an established cell line. *Biken J.* **10**, 37–39.

OKUNO, T. (1966). Problems of importance in an epidemiological research of the Thai hemorrhagic fever. *Nettai Igaku-kaiho* **7**, 33–39. (in Japanese).

OYA. A. (1965). Problem of Japanese encephalitis antibody. *Sōgō-rinsho* **14**, 1237–1240. (in Japanese).

PORTERFIELD, J. S. (1960). A simple plaque-inhibition test for the study of arthropod-borne viruses. *Bull. WHO.* **22**, 373–380.

SNEDECOR, G. W. (1946). Statistical methods applied to experiments in agriculture and biology. The Iowa State College Press.

WISSEMAN, C. L. JR., KITAOKA, M. and TAMIYA, T. (1966). Immunological studies with group B arthropod-borne viruses. V. Evaluation of cross-immunity against type 1 dengue fever in human subjects convalescent from subclinical natural Japanese encephalitis virus infection and vaccination with 17D strain yellow fever vaccine. *Am. J. Trop. Med. Hyg.* **15**, 588–600.