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SEROLOGICAL AND VIROLOGICAL STUDIES ON PATIENTS WITH DENGUE HEMORRHAGIC FEVER (DHF) IN CHANTHABURI PROVINCE, THAILAND[1]

I. SEROLOGICAL STUDIES ON PAIRED SERA FROM DHF PATIENTS BY NEUTRALIZATION (N), HEMAGGLUTINATION INHIBITION (HI) AND STAINING TESTS

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SUMMARY Twenty paired sera were collected from DHF patients admitted to the provincial hospital of Chanthaburi, Thailand in 1978. They were tested by the N and HI tests and a newly developed staining test against all four serotypes of dengue (DEN) virus and Japanese encephalitis (JE) virus.

Four patients were demonstrated to be cases of primary DEN infection and the other 16 to be cases of secondary DEN infection.

The serotypes of recent DEN infections were easily determined by the N test in cases of primary infection, but were difficult to determine in cases of secondary infection because of the strong cross reactions among DEN serotypes. These cross reactions seemed to be due to subgroup-specific antibody, which reacts with all 4 serotypes of DEN virus in the N test.

However, acute phase sera from some patients with the secondary type of antibody response reacted monospecifically in the N test, indicating the serotype of the primarily infecting viruses.

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These serological studies, together with the results of virus isolation described in the accompanying paper, show that all 4 serotypes of DEN virus were present in the Chanthaburi area during the epidemic season of 1978.

INTRODUCTION

Since the epidemic of DHF was first reported in and near Bangkok (Hammon et al., 1960), many outbreaks of the disease have occurred in Thailand (Halstead, 1966).

The Chanthaburi province, about 300 km east of Bangkok, has been an endemic area of DHF, but few serological and virological studies on the disease have been done in this area. JE virus infection has also been reported in Thailand (Yamada et al., 1971; Edelman and Pariyanonda, 1973; Fukunaga et al., 1974), and since this is also a flavivirus, we tested sera for both DEN and JE viruses.

There have been many serological reports of studies on sera from DHF patients (Hammon and Sather, 1964; Russell et al., 1968; Nimmannitya et al., 1969; Halstead et al., 1969a; Gubler et al., 1979), but the N test, which is considered to be the most type-specific of all serological reactions (Hammon and Work, 1964), has scarcely been used in these studies, probably because the conventional method for the N test is too time-consuming for use in tests for all 4 serotypes of DEN virus in large numbers of specimens.

Recently we established a micro-system for rapid N tests for all 4 serotypes of DEN virus and JE virus, utilizing the peroxidase-anti-peroxidase (PAP) staining technique (Okuno et al., 1978). We also developed the 4 step PAP staining technique (Okuno et al., 1979) to detect the antibody reacting with virus antigens in cells infected with DEN viruses or JE virus. These methods seemed useful for serological and seroepidemiological studies on DHF. This paper describes the antibody responses in DHF patients in N, HI and staining tests and the possible differentiation of type-specific, subgroup-specific and group-specific antibodies in the sera of the patients.

MATERIALS AND METHODS

1. *Collection of paired sera*

Twenty paired sera were obtained from children who were diagnosed clinically as having DHF and who were admitted to Prapokklao Hospital, a provincial hospital in Chanthaburi. Acute-phase blood samples were taken on the day of admission and convalescent-phase samples on the day when the

TABLE 1. *Serological data on paired sera from DHF patients with primary DEN infection*

Serum No.		Age	Sex	Days after onset	N titer					
					DEN-1	DEN-2	DEN-3	DEN-4	JE	DEN-1
126	A[a]	8	F	(4)[c]	<20	<20	<20	<20	<20	<20
	C[b]				<20	32	120	30	<20	<20
143	A	9	F	3	<20	<20	<20	<20	<20	<20
	C			6	270	<20	<20	<20	<20	<20
176	A	0.5	M	6	<20	<20	<20	<20	<20	<20
	C			11	<20	400	<20	<20	<20	40
194	A	1	F	3	<20	<20	<20	<20	<20	<20
	C			6	1300	28	31	<20	<20	640

[a] Acute phase serum. [b] Convalescent phase serum. [c] ( ): Days between times when acute and convales-

children left hospital. Sera were separated from clotted blood and stored at −20°C for tests.

2. *Viruses*

Dengue viruses, type 1 (DEN-1) Hawaiian strain, type 2 (DEN-2) New Guinea B strain, type 3 (DEN-3) H-87 strain and type 4 (DEN-4) H-241 strain, and Japanese encephalitis (JE) virus, Nakayama strain, were used. The origins and passage histories of the DEN viruses are described elsewhere (Igarashi, 1978). JE virus was received from Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University. Stock viruses were prepared from 10% homogenates of infected mouse brain 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.

3. *Neurtralization (N) test*

The N antibody titers of test sera were determined by the PAP staining method as described previously (Okuno et al., 1978). Sera were heat-inactivated at 56°C for 30 min before use. Serially diluted test sera were mixed with a constant amount of virus suspension and incubated at 28°C for 2 h. Then the serum-virus mixture was inoculated onto BHK21 cells on Lab-Tek 8-chamber slides (Miles, Ill., U.S.A.). Virus controls were incubated with diluent only and run in parallel. Standard anti DEN or JE rabbit sera were used as positive controls in each experiment. After adsorption for 2 h, 0.4 ml of maintenance medium, consisting of 2% fetal calf serum (FCS) in MEM, was added to each well and the slides were incubated in a 5% CO_2 incubator at 37°C. After appropriate incubation periods, the slides were stained by the PAP method, and the stained foci were counted as described previously (Okuno et al., 1977). N titers were expressed in terms of the 50% focus reduction rate (FR_{50}), the reciprocal of the serum dilution that reduced the number of foci to 50% of the control value.

4. *Hemagglutination inhibition (HI) test*

Before the test, serum specimens were extracted with kaolin and absorbed with goose red blood cells. Tests were performed by the method of Clarke and Casals (1958) as modified for a microsystem (Sever, 1962), using antigens extracted from infected mouse brain with sucrose-acetone.

5. *Staining test*

For demonstration of serum antibodies that react with antigens in cells infected with DEN or JE virus, the 4 step PAP staining technique was used (Okuno et al., 1979).

Test sera were serially diluted 2-fold from 1: 400 to 1: 102,400 in phosphate buffered saline (PBS, pH 7.4). The diluted sera of DHF patients were dropped onto acetone-fixed BHK21 cells that had been infected with DEN or JE virus. Then the specimens were treated successively with the second (anti-human rabbit), third (anti-rabbit IgG sheep) and fourth (PAP complex) sera. Finally the peroxidase reaction was performed and the intensity of staining was examined under a light microscope. The reciprocal of the maximal dilution giving definite staining of the infected cells was defined as

HI titer				Staining titer				
DEN-2	DEN-3	DEN-4	JE	DEN-1	DEN-2	DEN-3	DEN-4	JE
<20	<20	<20	ND[d]	<400	<400	<400	<400	<400
80	160	80	ND	<400	<400	<400	<400	<400
<20	<20	<20	<10	<400	<400	<400	<400	<400
<20	<20	<20	<10	<400	<400	<400	<400	<400
<20	<20	<20	ND	<400	<400	<400	<400	<400
320	40	80	ND	<400	<400	<400	<400	<400
<20	<20	<20	ND	<400	<400	<400	<400	<400
40	40	20	ND	<400	<400	<400	<400	<400

cent phase sera were taken. [d] Not done.

the staining titer.

A staining titer of less than 1:400 was regarded as negative, because at higher concentrations specific staining of the infected cells was not detectable, owing to strong non-specific background staining.

RESULTS

Twenty DHF patients, 10 boys and 10 girls of 6 months to 13 years old, were tested. According to the criteria of Nimmannitya et al. (1969), four were classified as primary cases (Table 1) and the other 16 as secondary cases

TABLE 2. *Serological data on paired sera from DHF patients with secondary DEN infection*

Serum No.		Age	Sex	Days after onset	N titer					
					DEN-1	DEN-2	DEN-3	DEN-4	JE	DEN-1
108	A[a]	12	M	3	680	890	290	600	<20	320
	C[b]			8	18000	15000	8600	12000	49	≧10240
117	A	5	M	2	460	210	1800	510	<20	320
	C			5	2900	2400	34000	2400	<20	5120
118	A	4	F	3	<20	<20	<20	130	<20	20
	C			5	800	1400	800	2300	<20	1280
124	A	11	M	1	<20	<20	200	<20	<20	<20
	C			14	4700	2800	15000	5900	<20	5120
152	A	13	F	1	<20	230	<20	<20	<20	<20
	C			5	1700	12000	650	4300	20	2560
158	A	8	F	2	610	30	72	36	<20	160
	C			7	15000	9400	11000	14000	<20	≧10240
159	A	7	M	6	150	86	25	23	<20	80
	C			12	20000	22000	4600	3000	64	≧10240
161	A	8	M	4	130	86	80	100	<20	320
	C			9	6800	12000	6300	5300	<20	≧10240
177	A	3	M	4	32	96	220	80	<20	<20
	C			10	2100	2600	12000	700	<20	5120
179	A	12	F	3	1400	330	200	400	<20	640
	C			7	58000	12000	14000	34000	40	≧10240
183	A	1	M	4	78	700	54	230	<20	80
	C			8	9000	31000	6200	6000	36	5120
185	A	4	M	3	7400	6000	1200	630	<20	≧10240
	C			7	12000	14000	26000	7000	<20	2560
189	A	10	M	(4)[d]	1900	1500	240	740	<20	640
	C				23000	16000	16000	18000	<20	5120
191	A	11	F	5	320	2000	560	160	<20	640
	C			11	5300	11000	16000	590	<20	5120
202	A	10	F	2	8200	7600	12000	9800	30	≧10240
	C			11	7000	12000	8000	24000	24	≧10240
208	A	8	F	(6)	200	42	48	<20	<20	160
	C				30000	19000	13000	1800	<20	≧10240

[a] Acute phase serum. [b] Convalescent phase serum. [c] Not done. [d] ( ): Days between times when acute

(Table 2) of DEN infection from the results of HI tests. The days after onset of illness when the acute (A) and convalescent (C) phase sera were collected are shown in the tables.

1. *Primary DEN infection*

As shown in Table 1, serum specimens from cases No. 126, 143, 176 and 194 showed the pattern of primary antibody response in both the N and HI tests; that is, no antibodies in A phase sera were detectable at 1:20 dilution. However, some differences in the N and HI titers were demonstrated in the C phase sera. The N antibodies appeared to be more type-

HI titer				Staining titer				
DEN-2	DEN-3	DEN-4	JE	DEN-1	DEN-2	DEN-3	DEN-4	JE
640	640	1280	ND[c]	1600	1600	1600	1600	800
≧10240	≧10240	≧10240	ND	12800	12800	12800	25600	6400
640	640	640	ND	1600	1600	800	1600	1600
≧10240	≧10240	≧10240	ND	12800	12800	12800	12800	6400
160	80	160	ND	<400	<400	<400	<400	<400
≧10240	5120	≧10240	ND	1600	3200	3200	3200	3200
<20	40	20	ND	<400	<400	<400	<400	<400
≧10240	≧10240	≧10240	ND	12800	12800	12800	12800	3200
80	<20	20	ND	<400	<400	<400	<400	<400
≧10240	2560	≧10240	ND	3200	3200	3200	6400	1600
320	160	320	ND	<400	<400	<400	<400	<400
≧10240	≧10240	≧10240	ND	3200	3200	6400	6400	3200
320	160	320	ND	<400	<400	<400	<400	<400
≧10240	≧10240	≧10240	ND	12800	12800	12800	12800	6400
2560	320	640	ND	<400	800	<400	<400	<400
≧10240	≧10240	≧10240	ND	6400	6400	12800	12800	6400
<20	<20	<20	ND	<400	400	<400	<400	<400
≧10240	≧10240	≧10240	ND	12800	6400	12800	6400	6400
5120	320	640	ND	400	<400	<400	<400	<400
≧10240	≧10240	≧10240	ND	6400	3200	6400	6400	3200
640	160	640	40	<400	800	<400	400	<400
≧10240	≧10240	≧10240	1280	12800	12800	12800	12800	12800
≧10240	≧10240	≧10240	640	6400	3200	6400	3200	1600
≧10240	≧10240	≧10240	≧5120	≧25600	25600	≧25600	25600	12800
1280	1280	2560	80	<400	800	400	400	400
≧10240	≧10240	≧10240	640	6400	3200	6400	6400	25600
2560	640	2560	160	1600	<400	1600	1600	800
≧10240	≧10240	≧10240	1280	6400	6400	6400	6400	25600
≧10240	≧10240	≧10240	2560	6400	6400	12800	12800	25600
≧10240	≧10240	≧10240	≧5120	6400	6400	12800	12800	≧25600
160	160	160	10	<400	<400	<400	<400	<400
≧10240	≧10240	≧10240	2560	12800	6400	12800	6400	25600

and convalescent phase sera were taken.

specific than the HI antibodies, clearly indicating that the infecting serotype was DEN-1 for No. 143 and 194, DEN-2 for No. 176 and DEN-3 for No. 126. In addition, a strain of DEN-2 serotype was isolated from the A phase serum of case No. 176, coinciding with the result of the N test (see accompanying paper).

In case No. 143 no HI antibody was detected in either A or C phase serum, but N antibody against DEN-1 was demonstrated in C phase serum. This suggests that the N test is more sensitive than the HI test.

Staining tests on these paired sera all gave negative results, even though the C phase serum of case No. 194 gave an N titer of 1: 1300 with a relatively high HI titer (1: 640) against DEN-1 serotype. This suggests that type-specific antibody is not involved in the staining test.

2. *Secondary DEN infection*

As shown in Table 2, the N antibodies in the C phase sera of patients with secondary DEN infection were much higher than those in cases of primary infections and showed more cross-reactivity. Although the degree of cross-reaction of N antibody was less than that of HI antibody, it was difficult to determine the serotypes of recent infections.

In some cases, however, the A phase serum showed a N titer against one serotype in 4 serotypes of DEN virus. For example, No. 118 showed a N titer to DEN-4, No. 124 to DEN-3 and No. 152 to DEN-2. These results indicate that patients No. 118, 124 and 152 had had primary infections of DEN-4, DEN-3 and DEN-2 serotype viruses, respectively and had developed DHF by recent secondary infections with heterotypic DEN viruses. On the other hand, the C phase sera of these patients showed relatively high N antibody titers against all 4 serotypes. In these sera, two of the 4 serotypes of DEN virus must have been neutralized by antibodies (both type-specific and subgroup-specific) in the C phase serum induced by infection, and the other two serotypes must have been neutralized only by subgroup-specific antibody, which is known to cross-react with all 4 serotypes in the N test. It should also be noted that in all three cases, the highest N titers observed in the C phase sera were against the serotype considered to be that of the primary infection. This suggests a booster effect of secondary infection with different serotypes against the serotype of the virus in the primary infection.

The differences between the N titers and the other (HI and staining) antibody titers against JE virus were significant. The N titers of sera of patients with secondary DEN infection against JE virus were much lower than those against DEN viruses, whereas their HI and staining titers against JE antigens were as high as those against DEN antigens. This suggests that the HI and staining antibodies against JE antigen demonstrated with sera of patients with secondary DEN infection are due to group-specific antibody, which is known not to neutralize heterologous flaviviruses appreciably.

The staining titer demonstrated by the 4 step PAP staining technique seemed to correlate rather well with the HI titer.

DISCUSSION

In this study N tests showed that four patients with primary DHF were infected with DEN-1, DEN-2 or DEN-3 virus, as described above. In addition, a strain of DEN-4 serotype virus was isolated from an A phase serum of a patient (No. 124) with secondary DHF (see accompanying paper). Thus all four serotypes of DEN virus were present in the Chanthaburi area during the epidemic season of 1978.

As far as we know, this is the first report of N tests for all four serotypes of DEN virus and JE virus using paired sera of DHF patients. Results showed that the serotypes of recent secondary DEN infections cannot be determined by serological examinations, including the N test, except by virus isolation

and identification.

However, in this study, the antigenic relationships among group-specific (common to all flaviviruses), subgroup-specific (common to all 4 types of DEN viruses) and type-specific (specific to each of the 4 types of DEN viruses) antigens of DEN viruses (Schlesinger, 1977) were delineated from the view point of the antibody responses of DHF patients. When antibodies corresponding to these antigens are designated as group-specific (gs), subgroup-specific (sub-gs) and type-specific (ts) antibodies, gs and ts antibodies are defined as followings: gs antibody shows extensive cross-reaction in the HI and complement fixation (CF) tests with all flaviviruses (Sweet and Sabin, 1954; Casals and Brown, 1954) while ts antibody reacts with only one of the four serotypes of DEN viruses in the N test (Sabin, 1950; Hammon et al., 1960; Russell and Nisalak, 1967). However, the immunological bases for separating the DEN viruses from other flaviviruses has remained uncertain, because the designation of DEN viruses as a distinct subgroup has rested primarily on clinical and other biological (host and vector spectrum) criteria (Schlesinger, 1977). Recently, De Madrid and Porterfield (1974) demonstrated by the plaque neutralization method using rabbit immune sera that the four DEN serotypes constitute one subgroup, showing no overlap with other flaviviruses.

In this work, the existence of gs antibody was demonstrated by both HI tests and staining tests with C phase sera of secondary DHF patients; that is, in both tests remarkable cross-reactions against JE virus antigens were observed, but this gs antibody did not neutralize JE virus appreciably.

The existence of ts antibody was demonstrated in the N tests with sera of primary DHF patients, which reacted specifically with only one DEN serotype (Table 1).

The sera of case No. 124 clearly demonstrated the existence of sub-gs antibody. This patient had had a primary infection of DEN-3 virus as demonstrated by the N test on his A phase serum, and had then been infected with DEN-4 virus as shown by virus isolation and identification (see accompanying paper). Nevertheless, his C phase serum showed high N titers against DEN-1 and DEN-2 serotype viruses. Since he had not been infected with DEN-1 or DEN-2, ts antibodies against DEN-1 and DEN-2 could not have neutralized these viruses. As mentioned above, gs antibody causes little neutralization, and thus the 2 serotypes of DEN virus with which this patient had not been infected must have been neutralized by sub-gs antibodies in the C phase serum. Although the N titers of his C phase serum against DEN-3 and DEN-4 were the sum of ts and sub-gs N antibodies, most of the N titer seemed to be due to sub-gs antibody, which neutralizes all DEN serotypes to almost the same extent as demonstrated with C phase sera after secondary infections. In other words, sub-gs antibody, which constitutes most of the N antibodies produced by secondary infection, masked the action of ts antibodies, thus making it difficult to determine the serotypes of secondary infection even by N tests.

Our data suggest that after primary DEN infection, a patient becomes " sensitized " not only to ts antigen of the infecting serotype but also to sub-gs and gs antigens, and on secondary infection with another DEN virus the sub-gs and gs antibody responses are induced to greater extents than the ts antibody response to the serotype of the recent infection.

In sera of secondary DHF patients, gs antibody against JE virus antigen was clearly demonstrated by HI and staining tests, but scarcely at all by the N test, consistent with the results reported by Igarashi et al. (1968). These data suggest that gs antibody cannot prevent infections by flaviviruses of different subgroups, as other workers have reported (Carey et al., 1965; Wisseman et al., 1966; Halstead et al., 1969b).

The staining titers in our study were consistent with those reported by Boonpucknavig et al. (1975) who examined antibodies in the

sera of DHF patients by the indirect fluorescent antibody technique. The C phase serum of case No. 194 showed a highly type-specific N antibody titer of 1: 1300 and also a relatively high HI titer (1: 640), but no detectable staining titer (<1: 400) against the DEN-1 serotype. This suggests that ts antibody is involved in the HI test, but very little in the staining test.

This work clearly shows the value of the N test in analysis of DEN virus infections. Further information, leading to a more correct understanding of DHF should be obtained by N tests not only with A and C phase sera, but also with those taken at various periods after infection, together with successful virus isolations and identifications.

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