

Antibody Responses Determined for Japanese Dengue Fever Patients by Neutralization and Hemagglutination Inhibition Assays Demonstrate Cross-Reactivity between Dengue and Japanese Encephalitis Viruses

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Received 2 January 2003/Returned for modification 25 February 2003/Accepted 12 May 2003

Titers of antibodies to infecting dengue virus serotypes determined by serum neutralization assay were higher than those of antibody to Japanese encephalitis (JE) virus in Japanese dengue patients after disease day 8. Titers of antibody to dengue virus antigens determined by hemagglutination inhibition (HI) assay were higher in only 1 of 23 serum specimens after disease day 11. Thus, the neutralization test is more reliable than the HI test for serological diagnosis of dengue in countries where JE vaccination is widely used or JE is endemic.

Dengue virus infections occur in most of the tropical and subtropical areas of the world, and dengue is considered one of the most important infectious diseases (2). Various serological techniques have been used for the laboratory diagnosis of dengue (4, 6, 7). Although immunoglobulin M capture enzyme-linked immunosorbent assay and reverse transcriptase PCR (RT-PCR) have been recently used for laboratory diagnosis of dengue (4, 5, 9), neutralization and hemagglutination inhibition (HI) tests are still frequently used in many laboratories. In Japan, most of the population is immune to Japanese encephalitis (JE) virus mainly because of JE vaccination and possibly occasional boost by JE virus. It is speculated that the presence of immunity to JE virus modulates immune responses induced by dengue virus infection. In the present study, we compared the titers of antibodies to the dengue and JE viruses determined by serum neutralization and HI assays in Japanese dengue patients.

Seventy-one serum specimens from 37 Japanese dengue patients were used. These serum specimens were obtained in clinics and hospitals in Japan from 1998 to 2001 and sent at 4°C to Department of Virology 1, National Institute of Infectious Disease, for laboratory diagnosis of dengue. The serum specimens were kept at 4°C before the assays. Disease days were defined as previously reported (9). Disease day 1 is the day of onset of disease, which is usually marked by fever. Dengue virus infection of these 37 patients was confirmed by detection of dengue virus genomes by PCR.

Focus reduction neutralization assays with peroxidase-antiperoxidase staining were performed as previously described by Okuno et al. (6). Vero cells were distributed at a concentration of 4×10^4 /well in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum

(FBS) to wells of 96-well flat-bottom plates (Corning Inc., Corning, N.Y.) and incubated in 5% CO₂ at 37°C for 1 day. Serum specimens were heat inactivated at 56°C for 30 min before use. Serum specimens were serially diluted fourfold from 1:10 to 1:10,240 in MEM containing 2% FBS. Forty-five microliters of each diluted serum specimen was mixed with an equal volume of dengue virus adjusted to give a final concentration of 100 focus-forming units per well. The serum-virus mixture was incubated at 37°C for 1 h. Twenty-five microliters of each mixture was transferred to wells containing Vero cell monolayers in 96-well flat-bottom plates, and the plates were incubated at 37°C for 1 h. The plates were washed once with phosphate-buffered saline. One milliliter of MEM containing 2% FBS was added to each well, and the plates were incubated at 37°C for 24 h for JE virus, for 40 to 48 h for the serotype DEN2 and DEN4 dengue virus strains, and for 56 to 72 h for the serotype DEN1 and DEN3 dengue virus strains. The cells were fixed with 100% ethanol and then stained successively at 37°C for 30 min each with anti-dengue virus or anti-JE virus rabbit serum diluted 1:1,000, anti-rabbit immunoglobulin G goat serum (ICN Pharmaceuticals, Inc., Aurora, Ohio) diluted 1:500, and peroxidase-antiperoxidase complex (ICN Pharmaceuticals, Inc.) diluted 1:10,000. The anti-dengue virus and anti-JE virus rabbit sera were prepared by immunizing rabbits with serotype DEN1 dengue virus and JE virus strain Beijing-1, respectively. 3,3'-Diaminobenzidine at 0.3 mg/ml in phosphate-buffered saline and 0.01% H₂O₂ were added, and the mixture was kept at room temperature for 5 to 10 min. Plates were rinsed with tap water and dried, and the foci were counted under a dissecting microscope. The neutralization antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to 50% or less of the control value.

Antibody titers were assessed by HI assay with 4 hemagglutinin units of dengue virus type 2 or 3 antigen as described by Clark and Casals (1). Infecting dengue virus serotypes were determined by RT-PCR. RT-PCR was performed as previ-

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TABLE 1. Neutralizing and HI antibody titers of dengue virus-infected patients

Patient no.	Age (yr)/sex ^b	Disease day	Serotype	PRNT50 ^c		HI titer		Ratio of DEN/JE	
				DEN ^b	JE	DEN	JE	Neutralization	HI
1	45/M	5	DEN1	80	<10	320 ^d	1,280 ^d	>8	1/4
2	29/M	5	DEN1	160	<10	80 ^d	640 ^d	>16	1/8
		15	DEN1	2,560	40	>20,480 ^d	>20,480 ^d	64	1
3	22/M	7	DEN1	160	<10	20 ^d	20 ^d	>16	1
		44	DEN1	1,024	<10	40 ^d	320 ^d	>102	1/8
4	24/M	4	DEN1	40	10	10 ^d	40 ^d	4	1/4
		9	DEN1	10,240	40	5,120 ^d	>20,480 ^d	256	>1/4
5	57/M	6	DEN3	10	40	1,280 ^d	10,240 ^d	1/4	1/8
		19	DEN3	10,240	40	10,240 ^d	>20,480 ^d	256	>1/2
6	25/M	1	DEN4	<10	<10	20 ^d	20 ^d	1	1
		18	DEN4	640	40	2,560 ^d	10,240 ^d	4	1/4
7	30/M	6	DEN1	10	<10	20 ^e	10 ^e	>1	2
		13	DEN1	160	10	320 ^e	640 ^e	16	1/2
8	40/M	7	DEN1	160	40	1,280 ^e	2,560 ^e	4	1/2
		13	DEN1	>640	160	10,240 ^e	>20,480 ^e	>4	<1/2
9	58/M	7	DEN1	10	10	320 ^e	640 ^e	1	1/2
		8	DEN1	160	40	640 ^e	5,120 ^e	4	1/8
10	35/F	8	DEN1	160	10	640 ^e	1,280 ^e	16	1/2
		19	DEN1	>640	40	2,560 ^e	5,120 ^e	>16	1/2
11	42/M	2	DEN1	<10	<10	10 ^e	<10 ^e	1	>1
		14	DEN1	160	<10	1,280 ^e	2,560 ^e	>16	1/2
12	54/M	5	DEN1	40	10	640 ^e	640 ^e	4	1
		12	DEN1	>640	40	>20,480 ^e	>20,480 ^e	>16	1
13	22/M	5	DEN1	<10	<10	10 ^e	<10 ^e	1	>1
		9	DEN1	40	10	320 ^e	320 ^e	4	1
14	22/M	4	DEN1	<10	10	320 ^e	40 ^e	<1	8
		16	DEN1	>640	40	5,120 ^e	>20,480 ^e	>16	<1/4
15	26/F	8	DEN1	40	<10	160 ^e	640 ^e	>4	1/4
		23	DEN1	160	<10	320 ^e	160 ^e	>16	2
16	26/F	7	DEN1	160	10	320 ^e	>20,480 ^e	16	<1/64
		16	DEN1	>640	40	>20,480 ^e	>20,480 ^e	>16	1
17	27/M	6	DEN1	<10	<10	40 ^e	<10 ^e	1	>4
		13	DEN1	640	10	5,120 ^e	5,120 ^e	64	1
18	27/F	4	DEN1	10	10	20 ^e	20 ^e	1	1
		7	DEN1	160	10	320 ^e	1,280 ^e	16	1/4
19	25/F	7	DEN1	10	10	80 ^e	10 ^e	1	8
		29	DEN1	160	10	160 ^e	160 ^e	16	1
20	30/F	2	DEN2	160	<10	40 ^e	20 ^e	>16	2
		9	DEN2	2,560	40	2,560 ^e	10,240 ^e	64	1/4
21	34/F	10	DEN2	160	<10	640 ^e	1,280 ^e	>16	1/2
		14	DEN2	640	<10	1,280 ^e	2,560 ^e	>64	1/2
22	23/F	5	DEN2	40	40	160 ^e	1,280 ^e	1	1/8
		9	DEN2	640	40	1,280 ^e	5,120 ^e	16	1/4
23	6/F	3	DEN2	<10	160	20 ^e	20 ^e	<1/16	1
		19	DEN2	640	160	2,560 ^e	10,240 ^e	4	1/4

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TABLE 1—Continued

Patient no.	Age (yr)/sex ^b	Disease day	Serotype	PRNT50 ^c		HI titer		Ratio of DEN/JE	
				DEN ^d	JE	DEN	JE	Neutralization	HI
24	24/F	3	DEN2	<10	10	80 ^e	40 ^e	<1	2
		8	DEN2	160	40	320 ^e	2,560 ^e	4	1/8
25	36/M	2	DEN2	<10	<10	80 ^e	40 ^e	1	2
		6	DEN2	>640	40	10240 ^e	>20,480 ^e	>16	<1/2
26	29/M	3	DEN2	<10	10	20 ^e	20 ^e	<1	1
		14	DEN2	160	10	5,120 ^e	10,240 ^e	16	1/2
27	22/F	4	DEN2	<10	<10	40 ^e	<10 ^e	1	>4
		11	DEN2	160	40	5,120 ^e	20,480 ^e	4	1/4
28	26/M	6	DEN2	160	40	160 ^e	320 ^e	4	1/2
		13	DEN2	>640	40	10,240 ^e	20,480 ^e	>16	1/2
29	51/M	4	DEN2	<10	<10	20 ^e	10 ^e	1	1
		9	DEN2	40	<10	40 ^e	10 ^e	>4	4
30	14/M	2	DEN3	<10	160	80 ^e	320 ^e	1/4	1/4
		9	DEN3	10,240	640	>20,480 ^e	>20,480 ^e	16	1
31	33/F	13	DEN3	160	10	2,560 ^e	5,120 ^e	16	1/2
32	21/F	2	DEN3	<10	<10	10 ^e	10 ^e	1	1
		9	DEN3	160	40	10,240 ^e	>20,480 ^e	4	<1/2
33	37/M	3	DEN3	<10	<10	40 ^e	10 ^e	1	4
		9	DEN3	160	10	2,560 ^e	5,120 ^e	16	1/2
34	40/F	2	DEN3	<10	<10	40 ^e	10 ^e	1	4
		19	DEN3	160	10	2,560 ^e	10,240 ^e	16	1/4
35	30/M	6	DEN3	40	<10	160 ^e	20 ^e	>4	8
		17	DEN3	160	10	320 ^e	640 ^e	16	1/2
36	25/F	13	DEN3	160	10	2,560 ^e	10,240 ^e	16	1/4
37	21/F	5	DEN3	<10	10	40 ^e	40 ^e	<1	1
		8	DEN3	160	40	1,280 ^e	2,560 ^e	4	1/2

^a M, male; F, female.

^b Titer of antibody to infecting dengue virus serotype determined by neutralization assay.

^c PRNT50, 50% plaque reduction neutralization titer.

^d Antigen, DEN2 serotype.

^e Antigen, DEN3 serotype.

ously reported (9). The sequences of the primers used to amplify each serotype of dengue virus and the target size were previously reported (3).

Serum specimens were collected twice from 34 of the 37 patients and once from 3 patients. Twenty-two serum specimens collected from 11 patients were examined for neutralizing antibody titers to all of the four serotypes of dengue virus. The titers of antibody to the infecting serotype were highest (data not shown). Therefore, titers of antibodies to the infecting dengue virus serotype and to JE virus determined by neutralizing test were compared for all of the serum specimens. Titers of antibody to dengue virus determined by neutralizing assay tended to be higher than those of antibody to JE virus (Table 1). Titers of antibody to infecting dengue virus serotypes determined by neutralizing assay were higher than those of antibody to JE virus in all of the 37 serum specimens collected after disease day 8 (Table 2).

The serum specimens were also examined by HI assay for titers of antibodies to dengue and JE virus antigens. Titers of antibody to JE virus antigen determined by HI assay tended to be higher than titers of antibody to dengue virus antigens (Table 1). Titers of antibodies to dengue virus antigens determined by HI test were higher in only 1 of 23 serum specimens collected after disease day 11 (Table 2). These results suggest that there is a reverse relationship between antibody titers determined by neutralization and HI tests in Japanese dengue patients.

Our results were consistent with the general understanding that the neutralizing antibody titer against the infecting dengue virus serotype is highest in a primary dengue virus infection. It is of interest that the titers of antibody to JE virus determined by HI assay were higher than those of antibody to dengue virus in most patients after disease day 11. The results suggest that examination of serum specimens by HI test alone may confuse

TABLE 2. Comparison between titers of antibodies to dengue and JE viruses

Disease days	Total no. of samples	No. of samples (%)					
		Neutralization antibody			HI antibody		
		DEN > JE	DEN = JE	DEN < JE	DEN > JE	DEN = JE	DEN < JE
1-4	16	2 (13)	9 (56)	5 (31)	8 (50)	6 (38)	2 (13)
5-7	18	12 (67)	4 (22)	2 (17)	5 (27)	3 (17)	10 (56)
8-10	14	14 (100)	0	0	1 (7)	2 (14)	11 (79)
11-13	8	8 (100)	0	0	0	1 (13)	7 (88)
14-16	6	6 (100)	0	0	0	2 (33)	4 (67)
17-19	6	6 (100)	0	0	0	0	6 (100)
>20	3	3 (100)	0	0	1 (33)	1 (33)	1 (33)

the serological diagnosis of dengue. Neutralization and HI tests are still widely used for dengue diagnosis in many laboratories (8). When serum samples are tested to confirm dengue virus infection in countries where JE vaccination is widely used or JE is endemic, it is recommended that a neutralization test, rather than an HI test, be performed to confirm dengue virus infection with serum samples collected after disease day 7.

We thank the clinic and hospital doctors who provided serum samples for laboratory diagnosis of dengue.

This work was supported by a grant for research on emerging and reemerging infectious diseases from the Ministry of Health, Labour and Welfare, Japan.

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