Quantitation of Dengue Precipitating Antibody by Inhibition Countercurrent Immunelectrophoresis

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The inhibition countercurrent immunelectrophoresis test was employed to detect dengue virus antibody in patients' sera. Anti-dengue type 2 titers determined by inhibition countercurrent immunelectrophoresis correlated well with hemagglutination inhibition titers. In secondary cases, more than fourfold increases in precipitating antibodies were observed. The control sera were negative except for sera from a few patients with systemic lupus erythematosus, which showed low titers. Simultaneous detection of dengue virus antigen and antibody in sera collected during the acute phase could confirm at least 90% of cases. This method is recommended as a routine technique to quantitate antibody in sera from suspected cases of dengue hemorrhagic fever.

The hemagglutination inhibition (HI) test described by Clarke and Casals (6) is the most common serological technique used for the diagnosis of dengue hemorrhagic fever (DHF). Other diagnostic procedures are the complement fixation test (11), the virus neutralization test (15), and virus isolation from patients' sera (8, 14). Immunofluorescence techniques have also been applied to determine titers of dengue antibody in patients' sera (2).

Countercurrent immunelectrophoresis has been widely used recently to detect hepatitis B surface antigen (HBsAg) (7), adenoviruses (9), influenza virus antibody (1), California group arboviruses (10), and dengue antigens in sera of DHF patients (4, 5).

CIE is simple to perform and requires no elaborate equipment. The technique can be applied to the routine examination of a large number of blood specimens. To apply the CIE technique for the determination of dengue antibodies in sera of DHF patients, the method of inhibition countercurrent immunelectrophoresis (ICEP) described by Milner et al. (12) was employed in combination with the CIE test.

MATERIALS AND METHODS

Patients' sera. Paired sera from patients with clinical diagnoses of DHF were obtained from the Bangkok Christian Hospital and the Chulalongkorn Hospital in Bangkok. Clinical diagnosis was based upon the criteria established by Nimmannitaya et al. (13). HI criteria for laboratory diagnosis of primary and secondary dengue virus infections were established by Winter and colleagues (16). Studies of 138 serum specimens from patients with clinical diagnoses of DHF

were carried out. A total of 4 patients were diagnosed as primary cases, and 52 patients were diagnosed as secondary cases of dengue virus infection. The remaining eight patients were non-dengue cases. All specimens were coded and run as unknowns. Control sera consisted of 30 specimens from normal adults, 20 from patients with lymphoma, and 20 from patients with systemic lupus erythematosus.

Dengue antigens. A suspension of 20% mouse brain infected with dengue type 2 virus (dengue-2) and having an HI titer of 1:320 was used as crude antigen.

Dengue antibodies. Rabbit antiserum to dengue-2 virus with an HI titer of 1:320 and a neutralizing titer of 1,520 was used throughout this study.

Methods. The ICEP test is based on the principle that dengue antibodies in the test serum will inhibit the precipitation of dengue antigen with homologous antibodies. Serial twofold dilutions of the test serum were incubated with a standard amount of dengue antigen (20% dengue-2) at 37°C for 1 h and at 4°C overnight. The test serum-antigen mixture was then reacted in a gel counterimmunelectrophoresis system against rabbit anti-dengue-2, using our previous protocol (4, 5). Briefly, 18 ml of 0.6% agarose in 0.05 M Veronal buffer, pH 8.2, was layered on a glass slide (3.25 by 4.0 in.; ca. 8.25 by 10.15 cm). Test sera, 25 μ l, were placed on the cathodal side in 4-mm wells, and the rabbit anti-dengue-2 serum was placed at the anodal side. The antigen and antibody wells were 3 mm apart. The electrode buffer was also 0.05 M Veronal buffer, pH 8.2. Electrophoresis was carried out at room temperature with a constant current of 20 mA per slide for 90 min. Results were read both without staining and with Coomassie brilliant blue staining for comparison.

If the patient's serum contained dengue antibodies, the ICEP test was positive; i.e., no precipitin band could be observed between the absorbed antigen and rabbit antiserum. If the serum did not contain dengue

antibody, the ICEP test was negative, and a precipitin line was observed. The endpoints of inhibition were read and interpreted as the titers of dengue antibodies in the test serum.

Twofold dilutions of the test serum from 1:2 to 1:512 were made in undiluted fetal calf serum. The CIE titer of rabbit anti-dengue-2 serum was determined before use in the ICEP test to select the optimal dilution of antibody which would give a consistently clear precipitin line. For this study, undiluted rabbit antiserum was used throughout.

RESULTS

Table 1 shows the HI and ICEP results for the four patients with primary dengue infection. The ICEP titers rose from <1:2 to 1:2 or 1:4 in all cases.

Results of HI and ICEP tests on acute- and convalescent-phase sera from 52 patients with secondary dengue infection are shown in Table 2. Acute-phase sera with HI titers of lower than 1:640 showed negative ICEP titers (Fig. 1). For acute-phase sera with HI titers of 1:5,120 and higher, ICEP titers were all positive. All convalescent-phase sera showed precipitating antibody by ICEP, with titers ranging from 1:8 to 1:512 (Fig. 2). At least fourfold increases in ICEP titers could be observed in all patients, except in case no. 31, 47, 48, 49, and 51, for whom HI and ICEP titers were already high in the acute phase. In these patients, titers increased only twofold or remained stationary. Case no. 52 showed a decrease in HI and ICEP titers.

Eight patients with clinical diagnoses of DHF, but who failed to show fourfold increases in HI titer, were classified as non-dengue cases, and negative ICEP titers were shown in both acute- and convalescent-phase sera. Sera from 20 normal adults showed no precipitating antibody by the ICEP test. Sera from 20 patients with lymphoma had HI titers ranging from <1:20 to 1:320, but all showed negative ICEP titers. Sera from 20 patients with systemic lupus erythematosus had HI titers ranging from <1:20 to 1:640. Sera from three patients with HI titers of 1:640 showed ICEP titers of 1:4. The remaining 17 cases had negative ICEP titers.

DISCUSSION

The results of this study indicated that ICEP could be applied to quantitate precipitating antibody to dengue virus antigen in sera from patients with primary and secondary dengue infections. Although only four cases of primary dengue infection were studied, all developed detectable precipitating antibody, and a low titer of precipitating antibody to dengue virus antigen was detected in one case during convalescence, when the HI titer was only 1:20. Patients without

TABLE 1. HI and ICEP dengue antibody titers of four patients with primary dengue infection

Case no.	Day of fever	Age (yr)	Phase at time of serum collection (hospital day)	Titer	
				HI	ICEP
1	5	2	Acute (1)	<1:20	<1:2
			Convalescent (15)	1:80	1:2
2	NK ^a	NK ^a	Acute (1)	<1:20	<1:2
			Convalescent (12)	1:160	1:2
3	6	2	Acute (1)	<1:20	<1:2
			Convalescent (8)	1:160	1:2
4	4	5	Acute (1)	<1:20	<1:2
			Convalescent (14)	1:20	1:4

^a NK, Not known.

HI antibody in the acute-phase specimen and with a titer between 1:20 to 1:160 in the convalescent-phase specimen were considered to have primary DHF. With similar criteria, these primary cases were confirmed by the ICEP test, since the acute-phase specimens showed an absence of antibody and the convalescent-phase specimens showed at least 1:2 ICEP titers.

In acute-phase sera from patients with secondary dengue infection, precipitating antibody was negative by ICEP in 46.1% (24 of 52) of sera with HI titers ranging from <1:20 to 1:2,560. When HI titers increased to 1:2,560, only 15.4% (2 of 13) of sera had negative ICEP titers. Sera with HI titers of 1:640 had ICEP titers ranging from 1:2 to 1:8, whereas sera with HI titers of 1:2,560, had ICEP titers ranging from 1:2 to 1:16. All sera with HI titers of 1:5,120 and higher had positive ICEP titers. Sera with higher HI titers also had higher ICEP titers, which indicated a good correlation of results obtained by the two techniques.

Since ICEP is an inhibition test, the acute-phase sera with negative ICEP titers (46.1%) were suspected to contain dengue virus antigen, and retrospective studies confirmed that this was the case. Using CIE for antigen detection and ICEP simultaneously gave the more satisfactory diagnostic results. Diagnosis could be done with most single specimens, and tests on paired sera confirmed the results. In cases when CIE and ICEP are both negative on acute-phase specimens, paired sera are necessary for diag-

TABLE 2. HI and ICEP dengue antibody titers of acute- and convalescent-phase sera from 52 patients with secondary dengue infection

Case no.	HI titers for following phase		ICEP titers for following phase		Case no.	HI titers for following phase		ICEP titers for following phase	
	Acute	Convalescent	Acute	Convalescent		Acute	Convalescent	Acute	Convalescent
1	1:40	1:1,280	<1:2	1:8	27	1:640	≥1:20,480	<1:2	1:256
2	<1:20	1:2,560	<1:2	1:128	28	1:1,280	1:2,560	1:2	1:32
3	1:40	1:5,120	<1:2	1:64	29	1:1,280	≥1:20,480	1:4	1:64
4	1:320	1:5,120	<1:2	1:256	30	1:2,560	1:2,560	<1:2	1:16
5	1:640	1:5,120	<1:2	1:32	31	1:2,560	1:5,120	1:16	1:32
6	1:640	1:5,120	<1:2	1:64	32	1:2,560	1:5,120	<1:2	1:64
7	1:640	1:5,120	1:2	1:64	33	1:2,560	1:5,120	1:4	1:64
8	1:640	1:5,120	<1:2	1:128	34	1:2,560	1:5,120	1:8	1:128
9	1:80	1:10,240	<1:2	1:128	35	1:2,560	1:10,240	1:2	1:64
10	1:160	1:10,240	<1:2	1:64	36	1:2,560	1:10,240	1:4	1:64
11	1:160	1:19,240	<1:2	1:128	37	1:2,560	1:10,240	1:4	1:64
12	1:640	1:10,240	1:8	1:64	38	1:2,560	1:10,240	1:2	1:128
13	1:640	1:10,240	1:2	1:128	39	1:2,560	1:10,240	1:16	1:128
14	<1:20	≥1:20,480	<1:2	1:512	40	1:2,560	≥1:20,480	1:4	1:128
15	1:40	≥1:20,480	<1:2	1:128	41	1:2,560	≥1:20,480	1:8	1:128
16	1:80	≥1:20,480	<1:2	1:128	42	1:2,560	≥1:20,480	1:4	1:256
17	1:160	≥1:20,480	<1:2	1:32	43	1:5,120	≥1:20,480	1:4	1:128
18	1:160	≥1:20,480	<1:2	1:128	44	1:5,120	≥1:20,480	1:32	1:128
19	1:320	≥1:20,480	<1:2	1:64	45	1:5,120	≥1:20,480	1:16	1:256
20	1:320	≥1:20,480	<1:2	1:128	46	1:10,240	1:10,240	1:16	1:128
21	1:320	≥1:20,480	<1:2	1:128	47	≥1:20,480	≥1:20,480	1:64	1:64
22	1:640	≥1:20,480	<1:2	1:64	48	≥1:20,480	≥1:20,480	1:128	1:128
23	1:640	≥1:20,480	1:4	1:64	49	≥1:20,480	≥1:20,480	1:128	1:128
24	1:640	≥1:20,480	<1:2	1:128	50	≥1:20,480	≥1:20,480	1:128	1:512
25	1:640	≥1:20,480	<1:2	1:128	51	≥1:20,480	≥1:20,480	1:512	1:512
26	1:640	≥1:20,480	1:8	1:128	52	≥1:20,480	1:2,560	1:128	1:32

FIG. 1. Correlation of HI and ICEP titers in acute-phase sera from 52 patients with secondary dengue infection.

FIG. 2. Correlation of HI and ICEP titers in convalescent-phase sera from 52 patients with secondary dengue infection.

nosis. These latter cases accounted for fewer than 10% of our total cases. For these patients, dengue virus antigen was found in acute-phase sera with HI titers of dengue-2 antibody of 1:5,120 or higher. Most of the patients with secondary dengue infection showed more than fourfold increases of ICEP titers in convalescent-

phase sera (Table 2). In case no. 31, only a twofold increase of antibody could be observed by HI and ICEP techniques. The acute-phase serum for this patient was collected 8 days after onset. The day of fever on which this serum was collected was not known, but possibly it was late in the course of infection so that both HI and ICEP titers were already high.

The specificity of the ICEP technique for detection of dengue virus antibody in patients with dengue infection was evidenced by the fact that eight patients with clinical diagnoses of DHF whose paired sera did not show fourfold increases of antibody by HI showed no antibody by the ICEP technique. ICEP was also negative for sera from 30 normal adults and 20 patients with lymphoma. Sera from three patients with systemic lupus erythematosus had HI titers of 1:640 and ICEP titers of 1:4. Systemic lupus erythematosus patients are known to have increased antibody titers to various viral antigens, possibly due to hyperactive immune responses of these patients (3). Therefore, the detection of dengue antibody in these patients was in accordance with results reported by other investigators. Since ICEP was negative in sera from patients with known diseases other than dengue infection and in normal human sera, with the exception of some systemic lupus erythematosus patients, ICEP titers of $\geq 1:8$ in acute-phase serum might be taken as evidence of dengue infection.

ICEP can be a practical test for routine laboratory use, since single or large numbers of specimens can be processed at a given time without much difference in time and expense. The HI test is a retrospective test, and paired sera are needed to make a diagnosis. Testing small numbers of specimens by HI is not practical or economical for most laboratories. Before the development of the ICEP test, physicians had to wait a long time for serodiagnosis of dengue infection, and after the patients had left the hospital by the time the diagnosis was confirmed. ICEP titers, which can be obtained in 1 day, could solve part of this problem. In our experience, a fourfold increase in the ICEP titer can be observed in only 2 to 3 days, which is quicker than increases in the HI titer and makes ICEP useful for rapid diagnosis.

Other advantages are the fact that extraction and absorption of test sera are not required for ICEP tests and that the antigen can be in a crude form instead of in the sucrose-acetone-extracted form needed for the HI test. Evaluation of the ICEP test confirms its value as one of the rapid diagnostic tests for DHF, especially from the standpoint of economy.

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