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Comparative Assay on Anti-dengue IgM Antibodies by the Indirect ELISA and IgM-capture ELISA

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Abstract: Anti-dengue IgM antibodies were measured by the indirect ELISA and IgM-capture ELISA using type 2 and 3 dengue antigens on dengue patients' sera. The assay results depended more strongly on the assay method than the serotype of dengue antigen used in the test. The results indicated that amounts of antibodies directed against virion and nonstructural viral antigen were not uniform in individual test serum.

Key words: Dengue, IgM-ELISA, virion, nonstructural viral antigen

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) have been major health problems in many tropical countries especially in southeast Asia (Halstead, 1966; 1980; 1992). Laboratory diagnosis on DF/DHF has been improved since the introduction of IgM-ELISA to determine recently infecting viral agent(Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989). It has generally been considered that the indirect ELISA, although its method is simple, could provide false negative results in the presence of high titered IgG class antibodies which compete with less amount of IgM antibodies to bind the assay antigen. However, as shown in the accompanying paper (Chew *et al.*, 1993), pretreatment of test sera with IgG adsorbents did not improve the efficiency to detect anti-dengue IgM antibodies in the indirect ELISA. On the contrary, such treatment apparently removed certain amount of anti-dengue IgM together with IgG antibodies, resulting in the reduced number of positive specimens. Theoretically, indirect ELISA using purified virion as assay antigen could detect or measure such antibodies that were directed against virion structural proteins. In the case of dengue and other flaviviruses, the envelope glycoprotein (E) is the most dominant antigenic component. While in the conventional IgM-capture ELISA (MAC-ELISA), assay antigens were either virus-infected cell culture fluid or extracted by sucrose-acetone method from infected mouse brain homogenate. When enzyme-conjugated detecting antibodies

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prepared from high titered DHF patients' sera (Innis *et al.*, 1989) were used in the MAC-ELISA with mouse brain-derived antigen, such a system could detect or determine not only the antibodies against virion structural protein but also those against nonstructural protein as well. It could be expected that the immune reaction in each individual patient may not be uniform and producing varying amount of anti-virion as well as anti-nonstructural viral antibodies.

In this communication, dengue patients' sera were assayed for their anti-dengue IgM antibodies by 2 different ELISA, indirect and MAC, using type 2 (D2) and type 3 (D3) antigens to see whether the assay result depends more on the assay method or serotype of dengue antigen used in the test.

Materials and Methods

Serum specimens: One hundred serum specimens of dengue patients were selected from the stock in the Division of Virology, Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. An aliquot of each serum was brought by the first Author to the Department of Virology, Institute for Tropical medicine, Nagasaki University. The sera were diluted 1:100 with PBS-T (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 and 0.02% NaN_3) for IgM ELISA. A pool of high-titered DHF patients' sera was also brought by the first Author and used to prepare anti-flavivirus IgG.

Preparation of purified dengue virion antigen and infected culture fluid: Dengue virions of type 2 (D2) New Guinea B strain and type 3 (D3) H87 strain were prepared from infected C6/36 cell culture fluid as described in the accompanying paper. A part of the infected culture fluid was kept aside to use as crude assay antigen in MAC ELISA.

Indirect IgM ELISA: The procedure was already described in the accompanying paper, except that not only D2 but also D3 antigen was used.

Preparation of anti-flavivirus IgG and conjugation with HRPO: The high titered DHF patients' serum pool was dialyzed against 100 volume of 5 mM sodium phosphate buffer, pH 7.0, at 4℃ overnight. The dialyzed serum was passed through No. 2 filter paper before loading to 20 ml volume of DEAE Sephacel (Pharmacia) which had been equilibrated with the same buffer. Fractions of 5 ml volume were collected by eluting the column with the same buffer and OD_{280} was measured for each fraction. Fractions showing high OD_{280} were pooled and dialyzed against 0.01 M sodium carbonate buffer, pH 9.0, and conjugated with HRPO by Wilson and Nakane's method (1978).

Indirect ELISA: The procedure and interpretation of the results were described in the accompanying paper.

MAC ELISA: The flat-bottom 96 well ELISA plate (Nunc) was coated with anti-human IgM (mu chain-specific, Cappel) at 1:200 dilution in a coating buffer (0.05 M sodium carbonate buffer, pH 9.6, containing 0.02% NaN_3) at 4℃ overnignt. The plate was inactivated by incubation with Blockace (Yukijirushi) at 37℃ for 1 hr. The plate was emptied and

washed successively 4 times with PBS-T using Microplate Washer (Titertek, M96V, Flow), and reacted with diluted test serum along with the standard positive and negative sera at 37℃ for 1 hr. The plate was emptied and washed as above, and assay antigen of infected C6/36 cell culture fluid was distributed to incubate at 37℃ for 1 hr. The plate was emptied and washed as above and HRPO-conjugated anti-flavivirus IgG was distributed and the plate was incubated at 37℃ for 1 hr. The optimal dilution of the HRPO-conjugated detecting antibody was predetermined by a checkerboard titration. The plate was emptied and washed as above and substrate solution (0.5 mg/ml *o*-phenylenediamine dihydrochloride and 0.02% H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0) was distributed. The plate was incubated at room temperature for 1 hr in the dark and the HRPO reaction was stopped by adding 1 N H_2SO_4. The OD_{474} of each well was recorded by an ELISA Analyzer (ETY-96, Tohyoh) using OD_{630} as a reference wavelength. The cut-off value of the MAC ELISA is the same as the indirect ELISA, that is double the average OD_{474} obtained for 8 wells of negative control. The ELISA OD of each test specimen was divided by the cut-off value to obtain P/N ratio. Any specimen with P/N ratio equal to or greater that 1.0 were considered as positive.

Statistical method: The methods described by Snedecor (1951), were used.

RESULTS

P/N ratio obtained by indirect and MAC ELISA using D2 and D3 antigens

All the test sera were examined for their anti-dengue IgM antibodies by the indirect and MAC ELISA, and the results were summarized in Fig. 1 and Table 1. In Fig. 1, each dot represents each test serum with their P/N ratio on the X-axis and Y-axis. It can be seen that significant correlation was observed between the P/N ratios obtained by the same assay method (indirect in Panel C, or MAC in Panel F), even when the serotype of assay antigen is different (D2 or D3). The correlation coefficient (R) was 0.455 for the indirect (Panel C), and 0.360 for MAC ELISA (Panel F), respectively. On the other hand, almost no correlation was observed between the P/N ratio obtained by the same serotype of assay antigen when different assay methods were used. R value between indirect and MAC ELISA was 0.009 for D2 (Panel A), and 0.039 for D3 (Panel D), respectively. The R value was further lower for

Table 1. Average and standard deviation (SD) of P/N ratio of anti-dengue IgM antibodies obtained by indirect and MAC ELISA

Assay Antigen	Assay Method	P/N ratio	
		Average	SD
D2	MAC	2.113	2.068
D2	indirect	1.226	0.759
D3	MAC	1.176	0.774
D3	indirect	2.369	1.081

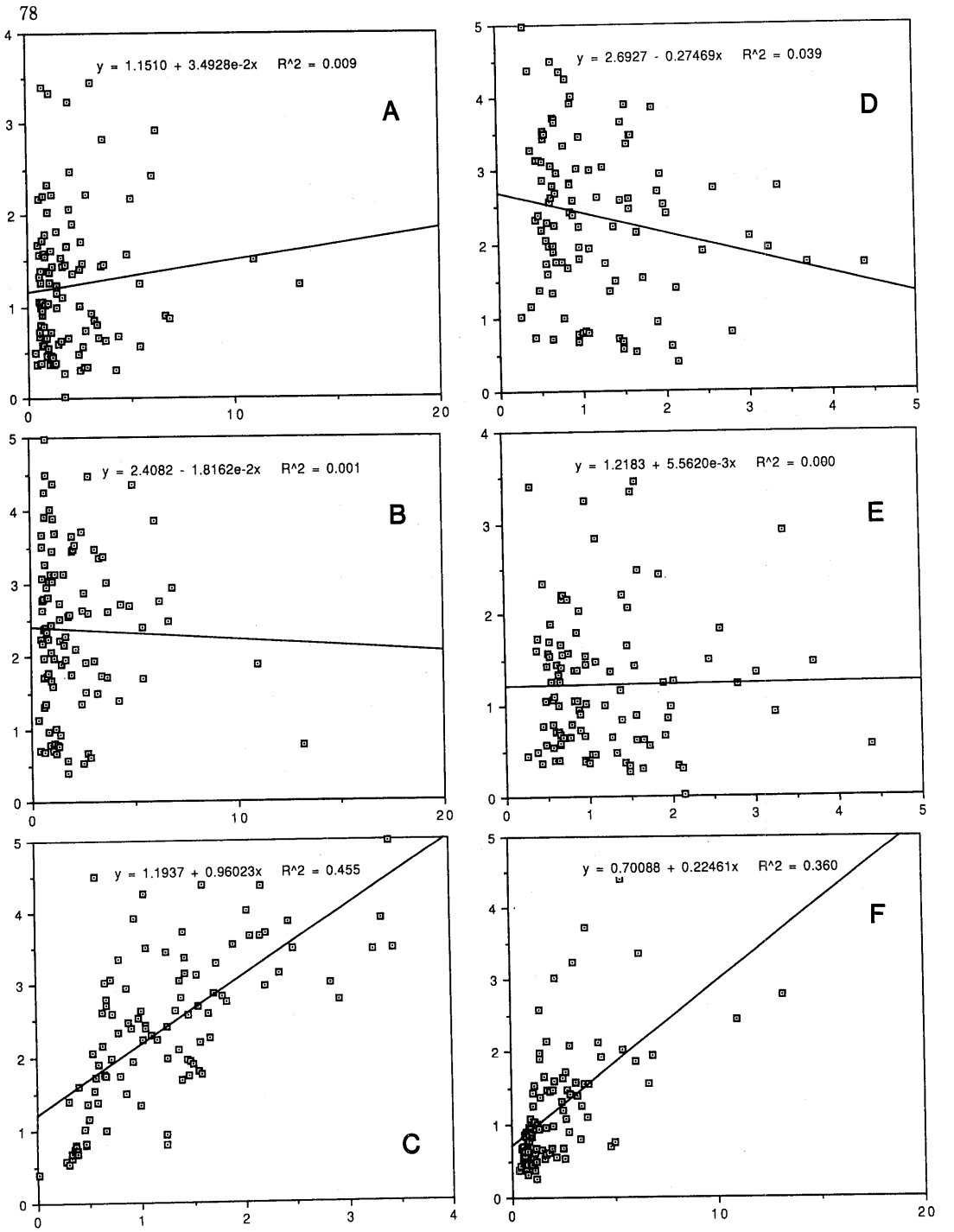

Fig. 1. Correlation between the P/N ratio of anti-dengue IgM antibodies obtained by indirect and MAC ELISA using D2 and D3 antigens
Each dot represents each test serum, with its P/N ratio obtained by indirect or MAC ELISA using D2 or D3 antigen. Panel A: D2 MAC on X-axis and D2 indirect on Y-axis; Panel B: D2 MAC on X-axis and D3 indirect on Y-axis; Panel C: D2 indirect on X-axis and D3 indirect on Y-axis; Panel D: D3 MAC on X-axis and D3 indirect on Y-axis; Panel E: D3 MAC on X-axis, D2 indirect on Y-axis; Panel F: D2 MAC on X-axis, D3 MAC on Y-axis, respectively. and MAC IgM ELISA.

the combinations of different assay method using different serotype of assay antigen [0.001 between D2 MAC and D3 indirect in Panel B, and 0.000 between D3 MAC and D2 indirect in Panel E, respectively]. The average and standard deviation (SD) of P/N ratio were calculated for each assay method and assay antigen as shown in Table 1. The statistical test (t-test) showed that the difference between the average P/N ratio was significant between following combinations: D2 MAC and D2 indirect ($P<0.01$); D2 MAC and D3 MAC ($P<0.01$); D2 indirect and D3 indirect ($P<0.001$).

Serodiagnosis by MAC and indirect IgM ELISA

The number of test serum which showed positive or negative results by the MAC and indirect IgM ELISA using D2 and D3 antigens was summarized in Table 2. The agreement was higher for the same assay method using different serotype of antigens [0.673 for D2 indirect and D3 indirect; 0.752 for D2 MAC and D3 MAC] than the different assay method using the same serotype of antigen [0.475 for D2 indirect and D2 MAC; 0.366 for D3 indirect and D3 MAC]. Further reduction of the agreement was not observed for the combinations of

Table 2. Number of positive specimens obtained by the indirect and MAC ELISA using D2 and D3 antigens and agreement of the test results

Compared results, assay antigen and method	Number of specimens		Agreement ratio
	observed	agreed	
D2 MAC (+); D2 indirect (+)	35	48	0.475
D2 MAC (−); D2 indirect (−)	13		
D2 MAC (+); D2 indirect (−)	31		
D2 MAC (−); D2 indirect (+)	13		
D2 MAC (+); D3 indirect (+)	54	58	0.574
D3 MAC (−); D3 indirect (−)	4		
D3 MAC (+); D3 indirect (−)	12		
D3 MAC (−); D3 indirect (+)	31		
D2 indirect (+); D3 indirect (+)	54	68	0.673
D2 indirect (−); D3 indirect (−)	14		
D2 indirect (+); D3 indirect (−)	2		
D2 indirect (−); D3 indirect (+)	31		
D2 MAC (+); D3 MAC (+)	42	76	0.752
D2 MAC (−); D3 MAC (−)	34		
D2 MAC (+); D3 MAC (−)	24		
D2 MAC (−); D3 MAC (+)	1		
D2 indirect (+); D3 MAC (+)	21	44	0.436
D2 indirect (−); D3 MAC (−)	23		
D2 indirect (+); D3 MAC (−)	33		
D2 indirect (−); D3 MAC (+)	24		
D3 MAC (+); D3 indirect (+)	32	37	0.366
D3 MAC (−); D3 indirect (−)	5		
D3 MAC (+); D3 indirect (−)	11		
D3 MAC (−); D3 indirect (+)	53		

different assay method using different serotype of antigen [0.574 for D2 MAC and D3 indirect; 0.436 for D2 indirect and D3 MAC].

DISCUSSION

Our results showed that the result of anti-dengue IgM ELISA was influenced more significantly by the assay method (MAC or indirect) than by the serotype of assay antigen (D2 or D3). Such a result indicates that in some patients' sera IgM antibodies were preferentially produced against virion antigen, probably E protein, while, in other patients antibodies directed against nonstructural protein may predominate. This reasoning waits verification by further experiments using other methods, such as western blotting on several test sera which showed significantly different P/N ratios by MAC and indirect ELISA. In the conventional western blotting, however, it should be kept in mind that the assay antigen has to be denatured before loading to the SDS-PAGE, and the procedure may miss to detect such antibodies directed to the conformational-dependent epitopes. Nevertheless, it may be interesting to see whether the different reactivity of the patient's sera to different viral antigenic components may be related with the clinical manifestation of DF/DHF, since immunopathological processes have been postulated to play important roles in the pathogenesis of severe DHF or dengue shock syndrome (Russell and Brandt, 1973; Halstead and O'Rouke, 1976; Halstead, 1988).

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