Production of Type 2 Dengue (D2) Monoclonal Antibody and Cell Culture Derived D2 Antigen for Use in Dengue IgM-Capture ELISA

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Abstract: In order to strengthen laboratory diagnostic capability on dengue by IgM-ELISA in Malaysia, type 2 dengue (D2) monoclonal antibody (MAb) and cell culture-derived D2 antigen were prepared as culture fluid of hybridoma cell line and D2-infected C6/36 culture fluid, respectively. Comparative IgM-ELISA was carried out on 90 serum specimens from dengue patients using newly prepared reagents in comparison with routine reagents (D2-MAb mouse ascitic fluid from CDC, USA, and D2 antigen extracted from infected suckling mouse brains). The newly prepared MAb at 2-10 fold higher concentration than that estimated by antigen detection ELISA, in combination with the undiluted D2-infected C6/36 cell culture fluid, provided comparable sensitivity (93.3-97.9%) to that of routine reagents (90.0-98.5%).

key words: Dengue, IgM-ELISA, monoclonal antibody, antigen

INTRODUCTION

Dengue virus infection has become a serious health problem in the tropics worldwide, because of (1) increasing number of patients, (2) enlarging epidemic areas, and (3) appearance of severe clinical manifestation: dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (World Health Organization, 1966; Halstead 1966, 1980, 1992, 1993). The laboratory diagnosis on viral infection is important in order to provide accurate information to the clinicians for proper case management, and to the epidemiologists and health administrators for efficient control measures (Igarashi, 1994). In the laboratory diagnosis on dengue, classical hemagglutination-inhibition (HI) test (Clarke and Casals, 1985; Shope and Sather, 1979; World Health Organization, 1986) has gradually been replaced by or supplemented with IgM-ELISA (Burke, 1983; Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989). In the Division of Virology, Institute for Medical Research (IMR), Malaysia, dengue IgM-ELISA has been used for routine diagnosis following the method of Lam *et al.* (1987). One of its 2 key reagents, D2-MAb, was supplied by the

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courtesy from the Centers for Disease Control and Prevention (CDC), USA, and another reagent has been produced from infected suckling mouse brains by classical extraction procedures (Clarke and Casals, 1958), which is laborious, time consuming and requires animal handling and large volume of organic solvent.

Strengthening of the diagnostic capability on dengue and Japanese encephalitis was planned among several objectives in the 3 years medical cooperation project by the Japan International Cooperation Agency (JICA) which has been carried out at IMR since 1993. The production of diagnostic reagents was listed under this objective, in order to improve current diagnostic methods and distribute them to local laboratories in the future. Production of above-mentioned 2 key reagents was apparently a limiting factor for the achievement this objective.

Our previous communication (Mohamed *et al.,* 1995b) showed that dengue cross-reactive MAb (MF4/5A5/C3) and a cocktail of 4 dengue serotype antigens as infected cell culture fluids can successfully be used in the IgM-ELISA with better sensitivity and similar specificity compared with the current reagents. Later, these 2 reagents were utilized to produce commercial dengue IgM-blot kit, and became difficult to get free of charge. In order to solve this problem, we tried to produce these 2 key reagents in the Division of Virology, IMR.

Materials and Methods

Virus: Dengue type 2 (D2) virus New Guinea B strain with multiple passages in C6/36 cell line was used in the experiments. The seed virus was aliquoted and stored at −70℃.

Cell culture: The C6/36 cell line (Igarashi, 1978) was grown in screw-capped plastic flasks at 28℃ with Eagle's medium in Earle's saline supplemented with 9% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids. The FCS concentration was reduced to 2% in the maintenance medium after virus infection.

The hybridoma cell line (3H5-1-21) which secretes D2-specific MAb (Gentry *et al.,* 1982; Henchal *et al.,* 1982) was kindly provided from Dr. Duane J. Gubler, Director, Vector-Borne Disease Division, CDC, Ft. Collins, CO, USA. The cell line was grown at 37℃ with the same medium as C6/36 cells but increasing the FCS concentration to final 20%.

Antigen detection ELISA: The micro sandwich method of Voller *et al.* (1878) was followed with some modifications, using the reagent volume of 100μl/well and reaction step for 1 hour at room temperature unless otherwise specified. The flat-bottom 96 well ELISA plate (Nunc, Denmark) was coated with antiflavivirus IgG diluted to 20μg/ml in the ELISA coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6, containing 0.02% NaN_3) at 4℃ overnight. The IgG was prepared from a pool of high titered DHF patient's sera by chromatography on DEAE-Sephacel column (Pharmacia, Sweden). The plate was inactivated with 4% skimmed milk in PBS (−), followed by washing with PBS-T (0.05% Tween 20 in PBS (−)) 3 times 3 minutes each. The test specimens, standard D2 antigen in serial 2-fold dilution, and mock-infected C6/36 cell culture fluid as negative control were distributed in duplicate wells. The plate was washed as above, followed by either of the 2 detection systems as described below.

In the detection system (1), the plate was reacted with antiflavivirus IgG which had been

conjugated with horseradish peroxidase (HRPO, Sigma, type Ⅵ, USA) according to Wilson and Nakane (1878). The optimal dilution of the HRPO-conjugate was predetermined by checkerboard titration as described below. The plate was washed as above and HRPO reaction was carried out using 0.05% *o*-phenylenediamine dihydrochloride and 0.01% H_2O_2 in 0.05 M citrate phosphate buffer, pH 5.0, in the dark. After stopping the HRPO reaction with 1N H_2SO_4, the ELISA OD at 492 nm was recorded with 630 nm as a reference wavelength. The ELISA titer of the test specimen was estimated by comparing its OD with those of serially diluted standard D2 antigen (Igarashi *et al.* 1981; Morita *et al.*, 1982). In the ckeckerboad titration, replicate wells were reacted with D2-infected C6/36 cell culture fluid as positive specimen (P), or mock-infected C6/36 cell culture fluid as negative specimen (N). After washing, the wells were reacted with HRPO-conjugated antiflavivirus IgG serially diluted in PBS-T, followed by washing and color development as described above. The optimal diluiton of the HRPO-conjugate was determined as such that provided the highest P/N ratio as well as the largest P-N difference in their ELISA OD.

In the detection system (2), the ELISA plate was reacted with anti-D2 MAb at appropriate dilution, followed by washing as above. The plate was then reacted with HRPO-conjugated anti-mouse IgG which is free from cross reaction to human IgG (Bio-Rad, USA). The plate was then washed, and color development was carried out as described above.

IgM-capture ELISA: The ELISA plate was coated with μ chain-specific anti-human IgM goat IgG (Cappel, USA) diluted 1: 200 in coating buffer at 4℃ overnight. The plate was inactivated as in the antigen detection ELISA, followed by washing. The plate was reacted with test sera or standard positive and negative sera at 1: 100 dilution in PBS-T. The plate was washed as above, and followed by the detection system (2) as described in the antigen detection ELISA. The HRPO-conjugated anti-mouose IgG was diluted to 1: 25,000 for the first test according to the instruction of the manufacturer. Since this dilution gave high background for the negative specimen, in the following experiments, the HRPO conjugate was diluted to 1: 100,000 which had been used for the routine test in IMR. Ninety test sera for IgM-ELISA were randomly selected from those examined by routine test in the Virology Division, IMR. The test specimen was scored as positive when its ELISA OD was equal to or greater than 2.0 of the negative control, according to Lam *et al.* (1978). Using this diagnostic criterion on the 90 test sera in the previous routine test, 66 specimens were scored as positive while 24 specimens were negative, respectively.

Result

Preparation of HRPO-conjugated anti-flavivirus IgG

Six independent conjugations were carried out and the optimal dilution of the preparations to distinguish positive (D2-infected C6/36 cell culture fluid) and negative (mock-infected C6/36 cell culture fluid) specimens were determined as described in the Materials and Methods. The results summarized in Table 1 shows the dilution to prepare working solution and the amount of the product. Although the dilution varied in a wide range from 1:25 to 1:6,400, the total amount of the final product was estimated to be sufficient for use of 1,500 ELISA plates.

Production of D2 antigen in the infected C6/36 cells

The ELISA antigen titer and volume of the specimens collected from D2-infected C6/36 cell cultures were summarized in Table 2. The antigen titer was not detectable in the infected cell homogenates which were prepared from secondarily harvested cells. The antigen titer in the secondarily harvested fluid from D2-infected cells incubated at 28℃ was not detectable in 2 harvests (19 and 23 September 1995), but another harvest on 16 February 1995 showed antigen titer of 15 ELISA unit. In contrast, D2 antigen titer between 12 to 16 ELISA units was reproducibly detectable in the culture fluid of D2 infected cells incubated at 37℃.

Table 1. Prearation of HRPO-conjugated anti-flavivirus IgG

Code	Optimal dilution	Number of aliquots 100μl each	Number of ELISA plates to be tested 10ml/plate
No. 1	1:400	42	168
No. 2	1:25	14	3
MH 6	1:6,400	17	1,088
MH 7	1:200	15	30
MH 8	1:200	54	108
MH 9	1:400	32	128
Total			1,525

Anti-flavivirus IgG was prepared from high titered DHF patients' sera and conjugated with HRPO using Wilson and Nakane's method (1978). The optimal dilution of the preparations was determined by the checkerboard titration as described in the Materials and Methods.

Table 2. D2-antigen from infected C6/36 cell culture specimens

Date of harvest	Incubation temperature and days	Harvest times*	Volume (ml)	ELISA titer
16 Feb 95	28℃, 7 days	2nd	7	15
16 Feb 95	37℃, 5 days	1st	44	14
12 Sep 95	37℃, 5 days	1st	80	16
16 Sep 95	37℃, 5 days	1st	120	16
19 Sep 95	28℃, 5 days	2nd	80	<1
19 Sep 95	28℃, 5 days	2nd cell	8	<1
21 Sep 95	37℃, 5 days	1st	80	16
21 Sep 95	37℃, 5 days	1st cell	8	<1
23 Sep 95	37℃, 5 days	1st	80	12
23 Sep 95	37℃, 5 days	1st cell	8	<1
23 Sep 95	28℃, 5 days	2nd	40	<1
23 Sep 95	28℃, 5 days	2nd cell	8	<1

*Infected culture fluid unless otherwise specified,
cell: infected cells solubilized in 1% Nonidet P-40 in PBS (−)
1st: harvest from primarily infected cells
2nd: second harvest from cells after harvesting 1st specimen

Production of D2-specific MAb in hybridoma cell culture fluid

Culture fluid was collected from hybridoma cell lines, and its dilution to prepare working solution in the antigen detection ELISA was measured using the detection system (2). In this test, D2 antigen or negative control antigen was first captured on the ELISA plate by anti-flavivirus IgG. After inactivation and washing, the plate was reacted with hybridoma culture fluids serially diluted in PBS-T. The plate was washed and reacted with a constant dilution of HRPO-conjugated anti-mouse IgG, followed by washing and color development. The dilution of hybridoma culture fluid harvested on different dates was estimated by comparing the ELISA OD of serially diluted test MAb with those of standard MAb supplied form CDC, USA, at its working dilution for routine test in IMR. The dilution and volume of each hybridoma culture fluid are summarized in Table 3. The results indicate that the total amount of hybridoma culture fluid is sufficient to make 28,000 ml working solution (or for 2,800 ELISA plates) for antigen detection ELISA.

IgM-ELISA using the C6/36 cell derived D2 antigen and D2 MAb from hybridoma cell culture fluid

Encouraged by the results shown in Tables 2 and 3, we carried out IgM-ELISA on 90 patient's serum specimens using new reagents (C6/36 cell derived D2 antigen and D2 MAb from hybridoma cell culture fluid). Previously carried out routine IgM-ELISA of these 90 test sera in IMR scored 66 positives and 24 negatives as described in the Materials and Methods. In the routine IgM-ELISA in IMR, the suckling mouse brain derived D2 antigen was diluted 1:100 to prepare working antigen solution. The antigen titer of this working solution was estimated as 4.4 ELISA units by antigen detection ELISA. Therefore, D2-infected C6/36 cell culture fluid with 16

Table 3. Production of D2-MAb as culture fluid of hybrodoma cell line (3H5-1-21)

Hybridoma culture fluid		Working solution	
Date of harvest	Volume (ml)	Dilution*	Volume (ml)
9 Jul 95	15	1:20	300
25 Jul 95	55	1:82	4,510
1 Aug 95	10	1:60	60
3 Aug 95	30	1:24	72
7 Aug 95	30	1:135	4,050
8 Aug 95	25	1:135	3,375
28 Aug 95	40	1:115	4,600
1 Sep 95	25	1:17	425
7 Sep 95	30	1:100	3,000
11 Sep 95	50	1:165	8,250
Total			28,642

*Estiamted from the chekcerboard titration in antigen detection ELISA as described in the Materials and Methods.

ELISA units antigen titer was diluted 1:2 to prepare working solution with antigen titer of 8 ELISA units. In this IgM-ELISA, detection system (2) was applied using D2-MAb in the hybridoma culture fluid which was harvested on 1 September 1995. This hybridoma culture fluid was diluted 1:16 according to its assay result as shown in Table 3.

IgM-ELISA on September 1995 using these diluted antigen and MAb showed relatively high background due to lower dilution (1:25,000) of HRPO-conjugated anti-mouse IgG. The number of specimens scored as positive in this test was 51 by the new reagents (cell culture derived D2 antigen at 1:2 dilution and hybridoma culture fluid at 1:16 dilution). Only additional single specimen was scored positive when cell culture derived D2 antigen was used in combination with MAb for routine test. There were only 2 more specimens which were scored positive when both D2 antigen and MAb for routine test were used. While, all 24 specimens which had been determined as negatives by routine test showed negative results by the new reagents. Therefore, the IgM-ELISA in this particular experiment possessed less sensitivity than routine test, although its specificity was high (Table 4).

In the repeat experiment on 21 September 1995, higher dilution (1:100,000) of HRPO-conjugated anti-mouse IgG was used in order to reduce the background, and concentration of D2 antigen and MAb was increased for better sensitivity. In this test, the number of positive specimens were 62 and 63 when hybridoma culture fluid was diluted to 1:8 and 1:4, respectively (sensitivity: 93.9%, and 95.5%). While the reagents for routine IgM-ELISA gave positive results for 60 specimens (sensitivity: 90.0%). The difference among sensitivity in these tests using

Table 4. Performance of IgM-ELISA using newly prepared reagents and routine reagents

Date tested	Antigen* & dilution	MAb** & dilution	Number of		Sensitivity***
			+ve	−ve	
20 Sep 1995	New 1:2	New 1:16	51	39	77.3%
	New 1:2	IMR	52	38	78.8%
	IMR	IMR	52	38	78.8%
21 Sep 1995	New 1:1	New 1:8	62	28	93.9%
	New 1:1	New 1:4	63	27	95.9%
	IMR	IMR	60	30	90.9%
22 Sep 1995	New 1:1	New 1:2	63	27	95.9%
	New 1:1	New 1:1	64	26	97.9%
	IMR	IMR	65	25	98.5%

*New antigen: D2 antigen prepared in D2-infected C6/36 cell culture fluid. IMR antigen: D2 antigen prepared from infected suckling mouse brains used at dilution for routine test in IMR.
**New MAb: culture fluid of hybrodoma cell line (3H5-1-21) which secretes D2-specific MAb. IMR: D2-specific MAb mosue ascitic fluid supplied from CDC, USA, diluted for routine test in IMR.
***Sensitivity of the test comapred with the IgM-ELISA on the same panel of 90 test sera which had been scored 66 positives and 24 negatives in the previous routine test. The test on 20 September 1995 gave high abckground due to lower dilution of HRPO-conjugated anti-mouse IgG, therefore sensitivity in this particular test was under estimated.

different reagests was not statistically significnat with confidence limit of greater than 95%.

In another repeat experiment on 22 September 1995, concentration of D2 MAb as hybridoma culture fluid harvested on 1 September 1995 was further increased, in combination with undiluted D2-infected cell culture fluid as antigen. In this test, the number of positive specimens was 63 and 64 when hybridoma culture fluid was diluted to 1:2 or undiluted, respectively (sensitivity: 95.5%, and 97.9%). While the reagents used for routine test detected 65 positive specimens (sensitivity: 98.5%). The difference in the sensitivity of the tests using new reagents and routine reagents was not statistically significant. There was a single specimen which had been scored as positive in the previous routine IgM-ELISA but gave negative result in the repeat experiment using routine reagents. This specimen might have been scored as false positive in the previous routine test or its IgM antibodies reacting to D2 antigen was inactivated during the storage period.

Discussion

Decentralizatoin of dengue IgM-ELISA is important for the rapid diagnosis which is essential for proper case management, epidemiological surveillance and control of dengue. The objective requires local production of key reagents: dengue antigen and MAb, in sufficient quantities by less laborious method.

This objective appeared to be easily achieved when the titers of D2-infected C6/36 cell culture fluid and D2-specific MAb in hybridoma culture fluid were measured in the antigen detection ELISA. However, the IgM-ELISA on serum specimens from dengue patients using these reagents showed that both antigen and MAb should be used at higher concentration than those estiamted in the antigen detection ELISA. This discrepancy may be due to the different sensitivity between antigen detection ELISA and IgM-ELISA. In the antigen detection ELISA, significant amount of test antigen was captured by the catching antibody (anti-flaivivirus IgG) and can be detectable with relatively low concentration of test reagents. In the IgM-ELISA on the other hand, anti-dengue IgM which had first been captured, then reacts with a smaller amount of D2 antigen. Therefore, much higher concentration of detecting reagents (D2 antigen and D2-MAb) may have been reguired to get positive reaction. Therefore the dilution of these 2 reagents should not be estiamted from the results of antigen detection ELISA. Independent test should be carried out by comparing the performance of IgM-ELISA using new and routine reagents on a panel of test sera. Production of higher concentration of D2 MAb will be achieved by preparing mouse ascitic fluid by D2 hybridoma cell line.

We have previously reported that increasing the incubation temperature of D2 or D3 infected C6/36 cells from 28℃ to 32℃ or 37℃ produced higher amount of viral antigen in the infected culture fluid (Mohamed *et al.,* 1985a). This observation was confirmed in the present experiment. In a separate study, we have shown that the increased D2 antigen production in the infected C6/36 cell line at elevated temperatures was accompanied by increased amount of intracellular as well as extracellular viral RNA (Mangada *et al.,* 1995). The study also showed that C6/36 cell line could grow better at 28℃ than at 32℃, and virtually negligible cell growth was

observed at 37℃. The mechanism of this dissociation between the optimal temperature for cell growth and virus growth remains to be investigated. For practical purpose to obtain sufficiently high titered D2 antigen, the infected C6/36 cell line should be harvested 5 days after incubation at 37℃. In our present study, 12−16 ELISA unit of antigen preparations were reporducibly obtained under these conditions. Further incubation of the infected cells under fresh medium or collecting infected cell homogenate did not appear useful for this purpose.

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