

Comparison of Dengue Virus Antigens in Sera and Peripheral Blood Mononuclear Cells from Dengue Infected Patients

Leera Kittigul, Nongyaow Meethien, Dusit Sujirarat¹, Chivat Kittigul², and Sirijit Vasanaavat³

Dengue virus antigens present in serum samples from the cases of secondary infection are usually low.¹ It requires replication of the virus and thus amplifies the antigenic components. Both *in vivo* and *in vitro* studies showed that peripheral blood leukocytes are a key site of dengue virus replication.²⁻⁶ The dengue antigens were demonstrated on the surface of mononuclear cells by specific fluorescent staining.⁷⁻⁸ Serological tests for detection of dengue IgM and IgG have been reported.⁹⁻¹² Nevertheless, the appearance of dengue IgM in sera was observed mainly after day 4 of illness in secondary infection.¹² The findings suggested that the rapid detection of dengue virus antigens could provide an early method for diagnosis of dengue infection.¹³⁻¹⁶ We therefore conducted a study to determine dengue virus antigens in sera compared with peripheral blood mononuclear cells (PBMC) from dengue infected patients by a biotin-streptavidin enzyme-linked immunosorbent assay (BS-ELISA).

SUMMARY The presence of dengue virus antigens in acute sera and peripheral blood mononuclear cells (PBMC) from dengue infected patients were determined by a biotin-streptavidin enzyme-linked immunosorbent assay (BS-ELISA). The frequency of the antigens detected in PBMC was higher than that in sera (53.8% vs 18.9%). In comparison with sera, the detection rate in PBMC was greater than six times: 7 cases were positive only in sera whereas 44 cases were positive only in PBMC, $p < 0.001$. The presence of the antigens in the sera did not depend on the severity of the disease, i.e. dengue fever, dengue hemorrhagic fever (grades I and II) or dengue shock syndrome (grades III and IV). In contrast, the presence of the antigens in PBMC increased from 36.8% to 100% when the infection was more severe. The dengue virus antigens could be detected in the samples collected between day 2 and day 7 after onset of the disease with the highest rate of detection (68.8%) in PBMC collected on day 4. The data suggest the use of PBMC with access to the appropriate acute-phase specimen for detection of dengue virus antigens.

MATERIALS AND METHODS

Patients, sera and PBMC

One hundred and six dengue infected patients and 72 non-dengue infected cases aged under 15 years were admitted at Khon Kaen Hospital, Khon Kaen Province between July and November, 1994. They were clinically diagnosed by physicians and laboratory confirmed for dengue infection by a standard hemagglutination inhi-

bition (HI) test.¹⁷ Dengue patients were classified as dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) following the criteria of WHO.¹⁸ Acute sera were collected on the same day with PBMC and

From the Department of Microbiology, ¹Department of Epidemiology, Faculty of Public Health, Mahidol University, ²Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, ³Khon Kaen Hospital, Khon Kaen Province, Thailand
Correspondence: Leera Kittigul

convalescent sera were collected from the patients before discharge from the hospital. The serum was separated by centrifugation for 10 minutes at 500 x g and stored at -70°C. Heparinized blood was collected from the patient on day 1 of admission. The leukocytes were separated by a Ficoll-Hypaque density floatation technique.¹⁹ Briefly, whole blood was diluted by the addition of an equal volume of normal saline solution. The blood was carefully layered over Ficoll-Hypaque (Isoprep®, Robbins Scientific Corporation, California) in round bottom tube and centrifuged at room temperature for 20 minutes at 800 x g. The top layer of plasma was pipetted off and the mononuclear cell layer was collected. The cells were washed 3 times by centrifugation for 15 minutes at 250 x g. Then, the PBMC were lysed by adding 5 mM phosphate buffer pH 8.0, mixed and stored at -70°C until used.

BS-ELISA

BS-ELISA was carried out in 96-well immuno microtiter plate (Nunc, Inter Med, Denmark) according to Egnund and Kjeldsberg²⁰ with some modification. In brief, the plate was coated with 100 µl per well of DEAE-cellulose purified human anti-flavivirus IgG diluted 1:400 in 0.05 M carbonate-bicarbonate buffer pH 9.6, and incubated overnight at 4°C. The DEAE-cellulose purified human anti-flavivirus IgG with or without covalent conjugation to biotin was prepared as described by Kittigul *et al.*¹² After washing with 0.15 M phosphate-buffered saline (PBS) pH 7.2, 100 µl of 2% bovine serum albumin (BSA) in PBS was

added to each well and incubated at 37°C for 1 hour. After washing with PBS containing 0.05% Tween 20 (PBS-T), 100 µl of serum or lysed PBMC diluted 1:100 in 1% BSA-PBS was applied to the well and incubated at 37°C for 1 hour. The plate was washed with PBS-T and 100 µl of biotinylated anti-flavivirus IgG diluted 1:800 was added. The reaction was incubated at 37°C for 1 hour and the plate was washed again. Streptavidin-horseradish peroxidase diluted 1:3,000 in 100 µl was added to each well and incubated at 37°C for 1 hour. After further washing, 100 µl of substrate solution containing 0.1 g/l of 3,3',5,5'-tetramethylbenzidine (TMB) in dimethylsulfoxide and 0.003% hydrogen peroxide in 0.1 M acetate buffer pH 6.0 was added and incubated at room temperature for 15 minutes. The color reaction was stopped by the addition of 100 µl of 4 N sulphuric acid. The absorbance was read at 450 nm using an automatic ELISA reader (EL 312e, Biotek, USA).

All assays included positive (2 wells) and negative (6 wells) controls. The positive and negative

samples were pooled sera with or without four-fold rising titers of HI antibodies obtained from Kittigul *et al.*¹² The positive and negative PBMC were pooled from dengue and non-dengue infected cases, respectively, confirmed by HI test. The cut-off positive BS-ELISA value was determined by calculation the mean of the optical density (OD) values of negative controls plus 2 standard deviations. The averaged OD of the serum or PBMC above the cut-off value was considered reactive for dengue antigens.

Statistical analysis

The distribution of qualitative variables in the detection of dengue antigens by BS-ELISA between acute sera and PBMC was compared using a McNemar test. A *p* value of < 0.05 was considered statistically significant.

RESULTS

All serum samples were confirmed by HI test. Among 106 cases with four-fold rising titers of HI antibodies, 1.9% had primary

Table 1. Sequence of dengue infection as determined by hemagglutination inhibition test, classified by dengue diseases

| Category of disease | Primary infection No. (%) | Secondary infection No. (%) | Total No. (%) |
|---------------------|------------------------------|--------------------------------|------------------|
| DF | 1 (0.95) | 18 (16.95) | 19 (17.9) |
| DHF I | 1 (0.95) | 31 (29.25) | 32 (30.2) |
| DHF II | - | 19 (17.9) | 19 (17.9) |
| DHF III | - | 33 (31.2) | 33 (31.2) |
| DHF IV | - | 3 (2.8) | 3 (2.8) |
| Total | 2 (1.9) | 104 (98.1) | 106 (100) |

dengue infection and almost of the patients (98.1%) had secondary infection, as shown in Table 1. Seventy two non-dengue cases without rising HI titers were enrolled in the experiment. Dengue virus antigens were detected by BS-ELISA in 18.9% of acute sera and 53.8% of acute PBMC from dengue infected patients. The negative results of both sample types were not different in non-dengue cases (Table 2).

Based on BS-ELISA, the presence of dengue antigens in sera were compared with those in PBMC from dengue cases. Seven cases were positive only in sera and 44 cases were positive only in PBMC. The detection rate of dengue virus antigens in PBMC was 6.3 times (44/7) greater than that of sera. There was statistically significant difference, $p < 0.001$ (Table 3).

Regarding the severity of dengue disease, the detection of antigens in serum samples was not obviously different among DF, DHF grade I, II and III except grade IV patients. In contrast, using PBMC, the positive cases increased from 36.8% to 100% when the infection was more severe (Table 4).

The dengue antigen positive detection rate in serum samples collected between day 2 and day 7 after onset of the disease increased gradually from 6.7% to 33.3% of the infected patients. The detection rate of the antigens in PBMC (from 33.3% to 68.8%) was higher than in sera. PBMC collected on day 4 gave the highest detection rate and dropped after the sixth day, as shown in Table 5.

Table 2. Detection of dengue virus antigens in acute sera and/or peripheral blood mononuclear cells (PBMC) of patients with or without dengue infection as determined by BS-ELISA

| Specimen | Dengue cases | Non-dengue cases |
|----------|---------------------------|---------------------------|
| | No. of positive/total (%) | No. of negative/total (%) |
| PBMC | 57/106 (53.8) | 69/72 (95.8) |
| Sera | 20/106 (18.9) | 70/72 (97.2) |

Table 3. Comparison between acute sera and PBMC samples for the detection of dengue antigens as determined by BS-ELISA

| Acute sera | Acute PBMC | | Total |
|--------------|----------------|----------------|------------|
| | Positive (No.) | Negative (No.) | |
| Positive | 13 | 7 | 20 |
| Negative | 44 | 42 | 86 |
| Total | 57 | 49 | 106 |

McNemar test; Chi-square = 25.412, $p < 0.001$

Table 4. Prevalence of dengue antigens in acute sera and PBMC from dengue cases with different disease severity

| Category of disease | Number of cases | Positive dengue antigens in | |
|---------------------|-----------------|-----------------------------|----------------|
| | | Acute sera (%) | Acute PBMC (%) |
| DF | 19 | 4 (21.1) | 7 (36.8) |
| DHF I | 32 | 4 (12.5) | 19 (59.4) |
| DHF II | 19 | 4 (21.1) | 10 (52.6) |
| DHF III | 33 | 6 (18.2) | 18 (54.5) |
| DHF IV | 3 | 2 (66.7) | 3 (100) |

Table 5. Prevalence of dengue antigens in acute sera and PBMC collected at various times after onset of the disease

| Day after onset of illness | Number of cases | Positive dengue antigens in | |
|----------------------------|-----------------|-----------------------------|----------------|
| | | Acute sera (%) | Acute PBMC (%) |
| 2 | 15 | 1 (6.7) | 7 (46.7) |
| 3 | 20 | 3 (15.0) | 10 (50.0) |
| 4 | 32 | 5 (15.6) | 22 (68.8) |
| 5 | 26 | 8 (30.8) | 11 (42.3) |
| 6 | 10 | 2 (20.0) | 6 (60.0) |
| 7 | 3 | 1 (33.3) | 1 (33.3) |

DISCUSSION

Dengue virus antigens were evaluated in sera compared with PBMC by BS-ELISA. The dengue antigens in PBMC (53.8%) higher than sera (18.9%) were registered since the viruses were associated with leukocytes in infected patients.²¹ They were isolated from peripheral blood leukocytes of experimentally infected rhesus monkey.²² Dengue virus replicated in PBMC cultures with peak titers at 48 hour.⁶ The virus bound to monocytes by dengue virus-antibody complexes and Fc-Fc receptor interactions with increased number of infected monocytes in secondary dengue infection.^{23, 24} Scott *et al.*²¹ could isolate dengue viruses from leukocytes approximately three times greater than plasma. However, Waterman *et al.*⁸ reported that virus isolation from peripheral blood leukocytes was less sensitive than that from serum or plasma and the detection rate of dengue antigens in leukocytes by fluorescent antibody testing was only 1/19 patients with confirmed dengue infection. The investigators explained

the difference in tissue culture technique and races of patients. In this experiment, PBMC alone permitted 6.3 times the detection rate of dengue antigens by BS-ELISA in comparison with serum samples.

The increased rates of dengue antigen detection in PBMC from patients with shock cases (DHF III and IV) suggested a possible relationship to the severity of infection. The antigens could be found in sera and PBMC starting from day 2 to day 7 after onset of the disease. The highest detection rate was observed in the PBMC collected on day 4. The results are comparable with the finding of the dengue viremia lasting approximately 5-7 days since isolation of virus was less frequent after the fifth day of disease and dropped off faster in plasma than peripheral blood leukocytes.²¹ Dengue virus was recovered from skin, lymph nodes and several leukocyte-rich tissues for up to 3 days after termination of viremia, and from circulating leukocytes only at the end of the viremic period. The amount of intracellular infection increased

toward the end of the viremic period, abruptly ending one to two days later. The phenomenon correlated closely with the time of onset of shock in dengue infection.²⁴ So far various diagnostic tests for dengue infection have been developed. The isolation and identification of dengue viruses are still time-consuming, laborious and insensitive. Detection of dengue antibodies by HI test requires paired sera in interpretation of the meaningful result. Although single serum is appropriate for IgM-capture BS-ELISA, IgM appears in serum 5-7 days after onset of the dengue disease.¹² Thus, the early and rapid diagnosis of dengue infection by antigen detection would help the physician in treatment of DHF and DSS promptly. The results suggest that PBMC may represent the important target sample for detection of dengue antigens rather than sera. The BS-ELISA procedure is rapid, simple, inexpensive and requires only single specimen although the sample preparation before assay and the optimal condition must be made carefully.

ACKNOWLEDGEMENTS

We thank Dr. Sataporn Meungin at Khon Kaen Hospital for assistance in clinical diagnosis of DF, DHF and DSS; the staffs of Pediatrics Ward, Out Patient Department and Laboratory Division, Department of Serology at Khon Kaen Hospital for specimen collection and laboratory service. This research was funded by a grant from Mahidol University.

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GENERAL INFORMATION

- DATE : 8-11 December 1998
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- LANGUAGE : English
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Atopic dermatitis, contact dermatitis,
urticaria, skin allergies
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