DETECTION OF DENGUE VIRUS ANTIGEN IN MONOCYTES TO SUPPORT THE DIAGNOSIS OF DENGUE HEMORRHAGIC FEVER

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ABSTRACT

Dengue virus infection is a major cause of morbidity and mortality in tropical and subtropical areas of the world. The immunopathological mechanism that results in severe complications of dengue virus infection, i.e. dengue hemorrhagic fever (DHF), is important to determine. Primary dengue infection induces serotype specific and serotype-cross-reaction. In secondary infections with a virus a different serotype from that which causes primary infections, the presence of cross-reactive non-neutralizing antibodies, results in an increased number of infected monocytes by dengue virus antibody complexes. This, in turn, results in marked activation of serotype cross-reactive CD4 and CD8 memory CTL and result in overproduction of cytokines that affects monocytes, endothelial cells, and hepatocytes. Platelets are destroyed by cross-reactive anti-platelets autoantibodies. Dengue virus induced vasculopathy and coagulopathy must be involved in the pathogenesis of hemorrhage and the unbalance between coagulation and fibrinolysis activation increases the likelihood of severe hemorrhage in DHF/DSS. Definite diagnosis of dengue is provided by the detection of virus in acute-phase sera of patients. Virus isolation can be accomplished with mosquito cell lines or mosquito inoculation. However, these methods are time consuming and labor intensive. The reverse-transcriptase polymerase chain reaction (RT-PCR) provides a potential means of rapid diagnosis, but requires specialized facilities and equipment as well as expensive. Therefore, a rapid, simple, sensitive and economic sera is needed for clinical and epidemiological investigations. An amplified immunocytochemistry examination using streptavidin–biotin is described for the detection the antigen of dengue virus on monocytes in patient's serum. Monocytes/macrphages are the major target cells for dengue virus and attachment of the dengue virus particle to the surface membrane receptor(s). This assay utilized biotinylated antibody directed against dengue antigens by anti-dengue complex monoclonal antibody and applied streptavidin peroxidase. After being incubated, 1-2 drops of DAB chromogen were added, counterstained and visualized by microscopy examination. The result was regarded as positive if the visualization was brown. The result of immunocytochemistry by streptavidin biotin was tested on 32 sera submitted routinely to our laboratory for confirmaton of dengue diagnosis. The sensitivity of the test streptavidin biotin was 88% and the specificity was 87.7%. In conclusion, the immunocytochemistry by streptavidin biotin can be used for early diagnosis of dengue infection.

Keywords: *dengue hemorrhagic fever, immunocytochemistry, streptavidin-biotin*

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INTRODUCTION

Dengue hemorrhagic fever (DHF) is a major health problem in Indonesia due to its capability in increasing morbidity and mortality. It is also the primary cause of treatment in hospitals and death in children (Gubler 1998; Sumarmo 1998). The pathogenesis of DBD and Dengue Shock Syndrome (DSS) remains controversial. The two most popular theories are "Secondary Heterologous Infection" or the occurrence "Antibody Dependent Enhancement (ADE)", which suggest indirectly that patients who have secondary infection with heterologous dengue virus serotype will have a higher risk of DHF/DSS. The clinical manifestation of dengue infection (DEN virus) is highly varied, from asymptomatic, unspecific mild fever, DHF to DSS (Nimmannitya 1993; Nimmannitya 1996; Sutaryo 1999; WHO 1997; Wuryadi 1999). DHF and DSS are the more severe type as compared to other types, and may cause shock as well as death (Halsteadt 1990; Malavige et al. 2004; Nimmannitya 1993). However, in the first stage, when the symptoms are less typical and difficult to be differentiated, it is difficult for the clinicians to establish the diagnosis and management (Faizi 1998).

The early diagnosis of DHF is established based on WHO criteria and conventional laboratory results (examination of thrombocyte count and hematocrit level). However, the results are not satisfactory. Supporting laboratory examination can be given by

determining antibody using hemagglutination-inhibitory test or anti-dengue IgM and IgG detection. However, obtaining positive results require time interval since IgM becomes positive after fifth day of fever. Positive results can also be detected for several months, in addition to the occurrence of cross-reaction with other types of flavivirus. Currently, the novel method to detect the causing virus is the use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). However, this method requires a high cost, particular instruments as well as certain skill to handle (Faizy 1998; Pangkalila 1997). This paper introduces a method for easy, quick, cost-saving and reliable diagnosis DHF by examining DEN virus antigen present at monocyte surface immunohistochemically using streptavidin-biotin.

MATERIALS AND METHODS

Thirty-two samples of blood from DHF-suspected patients aged more than 10 years visiting Kasih Ibu Hospital, Surakarta, from January to June 2004. The diagnosis was based on WHO criteria from the result of clinical examination by anamnesis and laboratory examination by blood evaluation, including thrombocyte count and hematocrit value. The specimen of vein blood was mixed with EDTA and divided into two parts, one for anti-dengue IgM and IgM examination, and the other was subjected for monocyte isolation. After the buffycoat (Periferal Blood Monocyte Cell/PBMC) was taken, a swab preparation was made finely on decaglass, like that during the making of peripheral blood preparation, and fixed with acetone and dried in air. After becoming dry, it was stained immunohistochemically with streptavidin-biotin and, after being added with hematoxylin, it was observed under the microscope. Positive results would reveal brown color.

RESULTS

The results of serologic examination from 32 DHF-suspected clinical patients had been obtained. To ease the statistical analysis, the patients were divided into three groups: first, comprising 18 DHF patients with positive anti-dengue IgM and IgG; second, comprising 7 DHF patients with positive anti-dengue IgM and negative anti-dengue IgG; and third, comprising 7 patients with negative anti-dengue IgM and IgG. From 32 patients, there were 6 (18.75%) female patients aged 10-20 years, 9 (28.125%) aged 21-30 years, 2 (6.25%) aged 31-40 years, and 3 (9.375%) aged 41-50 years. Male patients aged 10-20 years were 4 (9.375%), 21-30 years were 6 (18.75%), 31-40 years were 2 (6.25%), aged 41-50 years was only 1 (3.125%). From the data of DHF distribution based on age and sex, it is apparent that most of the patients aged 21-30 years, confirming the finding in the literature (Sugiyanto 2004) that the distribution of DHF patients aged more than 15 years was increasing. It is also apparent that the number of female patients was higher than that of male patients. This finding was different from that in the literature that both sexes have generally the similar ratio. Chan (1987) in Thailand found that female and male patients in the Philippines had a ratio of 1:1. Nimmanitya (1987) in Thailand found that, although severe cases were found more in women, it was not different statistically. Sutaryo (2004) found that there was no difference in the number of cases between male and female patients.

Table 1. DHF distribution, according to age and sex

Age	Female	Male
10 - 20 years	6 (18.75%)	3 (9.375%)
21- 30 years	9 (28.125%)	6 (18.75%)
31- 40 years	2 (6.25%)	2 (6.25%)
41- 50 years	3 (9.375%)	1(3.125%)
Total	20 (62.50%)	12 (37.50%)

Regarding its clinical symptoms, it is apparent that predominant clinical symptoms were continuous fever for less than 7 days, gastrointestinal abnormalities, such as nausea, vomiting, and bleeding that presented as positive RL, all of which supported findings the existing literatures. WHO (1997) suggests that the most common bleeding phenomenon is the positive torniquet test. According to Sutaryo (2004) the minimal bleeding manifestation is the positive torniquet test, while epistaxis bleeding is only found in several cases.

Table 2. Patient's distribution according to clinical symptoms

Clinical symptoms	Female	Male	Total
Febrile 1-2 days	19	6	25
Febrile 3-7 days	4	3	7
Myalgia/athralgia	17	8	25
Nausea, vomiting	15	7	22
Head pain	10	4	14
RL (+)	21	11	32
Epistaxis	3	1	4
Petechiae	5	1	6

In general, the result of serological test for detecting the presence of anti-dengue IgM and IgG in DHF-suspected patients using rapid diagnostic test has revealed the following results: From 32 DHF-suspected patients, 7 (21.875%) patients had positive anti-dengue IgM and negative anti-dengue IgG, 18 (56.25%) patients had

positive anti-dengue IgM and IgG, and the rest 7 (21.875%) patients had negative anti-IgM and IgG.

Table 3. Serologic results of anti-dengue IgM and IgG antibody in DHF-suspected patients

Results of serological examination of immunity	Female		Male		Total	
Anti-dengue IgG and IgM antibody	N	%	N	%	N	%
IgM (+) and IgG (-)	5	15.6	2	6.2	7	21.9
IgM (+) and IgG (+)	11	34.4	7	21.9	18	56.2
IgM (-) and IgG (-)	5	15.6	2	6.2	7	21.9
Total	21	65.6	11	34.4	32	100

Table 4 shows the result of anti-dengue IgM and IgG serologic test and the result of immunohistochemical test with streptavidin-biotin. To find whether the result of immunohistochemical test can be used as a reliable diagnostic test for DHF-suspected patients, the result of the test should be subjected to statistical test of the three groups. Statistical test used was chi-square test with the p value of less than 0.01.

Table 4. Result of immunohistochemical test with streptavidin-biotin in DHF clinical patients with the result of anti-dengue IgM and IgG serologic test

Virus antigen detection	IgM(+)IgG(-), IgM(+) IgG(+) patients	IgM(-) and IgG (-) patients	Total
SB immuno test (+)	22	1	23
SB immuno test (-)	3	6	9
	25	7	32

To find whether immunohistochemical test with streptavidin-biotin to detect dengue virus antigen on monocyte surface could be used as supporting facility for DHF diagnosis, a sensitivity and specificity test was needed. The standard of comparison was the result of clinical diagnosis according to WHO criteria supported with anti-dengue IgM and IgG serologic examination. The result of sensitivity and specificity test can be seen in Table 5.

Table 5. Results of immunocytochemistry test in DHF clinical patients with anti-dengue IgM and IgG serological test.

Immunocyto-chemical test	DHF patients in line with comparative standard	Febrile not in line with comparative standard	Total
Immuno test (+)	22	1(2)	23
Immuno test (-)	3	6(12)	9
Total	25	7(14)	32

True positive = 22, false positive = 1,
true negative = 6, false negative = 3
( ) = correction factor

The sensitivity and specificity as diagnostic test used the formula 2 x 2, which can be seen in Table 5, with the estimation as follows: sensitivity 22/25 X 100% = 88%, and specificity 12/14 X 100% = 85.71%.

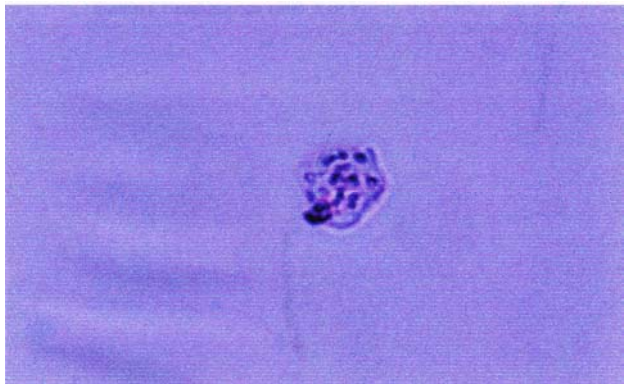

Figure 1. Negative result of DEN virus detection in monocytes with immunocytochemical examination with streptavidin-biotin (magnification 3000)

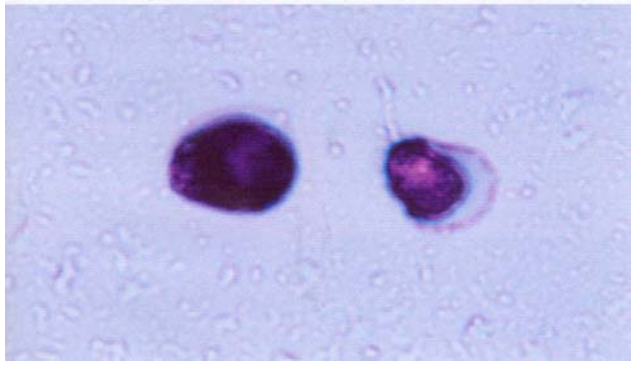

Figure 2. Positive result of DEN virus detection in monocytes with immunocytochemical examination with streptavidin-biotin (magnification 3000)

DISCUSSION

DHF remains a major health problem in Indonesia. Although the mortality rate has been reduced, in general the number of cases tends to increase from one year to another (Suroso 1997). The manifestation of the disease is highly varied, from the mildest to the most severe, which can be accompanied with shock (Soewandoyo 1997; WHO 1986). Patients who did not show DHF clinical symptoms is the majority, and this group is epidemiologically significant since they can serve as the source of transmission to other persons around them (Syahrurahman et al. 1993). Similarly, in the other

group, DHF-suspected clinical patients, if the diagnosis is not established as early as possible, the disease can lead to severity, susceptible to shock, and finally may result in fatality due to DSS. Therefore, the definitive diagnosis of DHF is highly important, since, in addition to assist the treatment and management of DHF patients, it also helps the prevention program in the community (Syahrurahman et al. 1993). Recently, DHF diagnosis is based on WHO criteria and the result of conventional laboratory examination supported with antibody determination using hemagglutination test or anti-dengue IgM and IgG detection. Theoretically, if all the results of examination have been available, DHF diagnosis can be established. However, in fact, these results cannot be immediately completely available. Sometimes it takes until several days and the specificity and sensitivity to detect anti-dengue IgM and IgG are often inadequate. It depends on the level of the disease, condition, the virus' type/serotype as well as its virulence (Wuryadi 1999).

Various studies have been conducted to create a new effort for establishing DHF definitive diagnosis, such as culture examination to isolate the causing virus or by means of PCR hybridization technique. However, although both methods can be used to determine the definitive diagnosis, its application still faces various obstacles. In addition to lengthy time for obtaining the result, it also requires sophisticated instruments, which results in higher cost, while its positivity values are highly varied (Myagotovich et al. 1997). Furthermore, if an outbreak occurs in an area, generally the diagnosis is established based on clinical and serological symptoms (anti-dengue IgM and IgG detection), while the results are often not promising. This is because when the patient arrives at the hospital, the antibody has possibly not been formed, or positive results may originate from previous disease or be caused by other flavivirus infection. This study detected virus antigen existing in vitro on monocyte surface by using anti-dengue complex monoclonal antibody. Since the DEN virus enters the body through *Aedes aegypti* mosquito bite, during incubation period the virus enters the circulation and viremia occurs. The occurrence of viremia in dengue infection is short, and the primary target of dengue virus is monocytes and dengue virus will be captured by monocytes since the latter have receptor on their surface (Chen YC 2002; Juffrie et al. 2000).

Table 1, based on age and sex, it can be seen that DHF can affect all age groups and sex, and statistical test reveals no significant difference. In this study the author recruited DHF patients aged more than 10 years as it had correlation with 10 cc blood sample taking, because children were hard to be cooperative without the presence of their own parents. However, the results of this study were apparently in line with those in the literature (Gubler et al. 1992; Soemarmo 1992; Tongcharoen 1993) suggesting that adult DHF patients were increasing. This study found 4 patients in advanced age (more than 40 years old). This study also showed that female patients were more than male patients. According to Sugiyanto (2004), significant difference was ever found between male and female pediatric patients. The same author also stated that there were reports from several countries that many groups of female with DSS had higher mortality rate than male patients. Based on clinical symptoms, the symptoms of fever was found in 32 patients (100%), vomiting in 22 (68.75%), positive tourniquet test in 32 (100%), myalgia in 25 (78.125%), and head pain in 14 (43.75%). These symptoms are often found in DHF patients, and statistical test revealed no significant result, so that clinical symptoms cannot be used as a supporting tool for establishing DHF diagnosis. Therefore, it needs other supporting diagnostic tools. For establishing DHF diagnosis, clinical examination followed with serological test for confirmation (anti-dengue IgM and IgG examination) is a routine serological test carried out by clinicians in hospitals. If the anti-dengue IgM and IgG are positive, the patient has primary or secondary DHF, but if the anti-dengue IgM is positive and anti-dengue IgG is negative, the patient has primary DHF, while if both anti-dengue IgM and IgG are negative, the patient has other non-DHF fever.

Based on those data, the author performed hypothetical test using immunohistochemical assay with streptavidin-biotin to those suspected DHF patients. The result of examination in 32 DHF-suspected patients showed that the serum of 23 DHF patients (71.88%) had positive streptavidin-biotin test, and that of 9 DHF patients (28.12%) had negative streptavidin-biotin test. The result of streptavidin-biotin test to the serum of 7 non-DHF patients showed that 5 patients (71.43%) had negative, and 2 patients (28.57%) had positive results. Furthermore, from 25 serum samples from DHF patients compared with 7 serum samples from non-DHF patients, using chi-square test it was found that there was significant difference. This indicated that immunohistochemical test using streptavidin-biotin was able in differentiating DHF and non-DHF patients. To find whether it is also a reliable test for to use as DHF diagnostic tool, the streptavidin-biotin test should also be tested for its validity.

To find the diagnostic validity, the result of virus antigen detection in monocyte surface using immunohistochemical examination with streptavidin-biotin was compared with sample group of DHF patients according to WHO criteria, supported with the result of serologic examination of anti-dengue IgM and

IgG antibody with other non-DHF patients as non-healthy control. Positive anti-dengue IgM and IgG was found to have sensitivity of 88% and specificity of 85.71%. The result of serum examination in 25 patients using immunohistochemical test with streptavidin-biotin revealed negative result in 3 patients (12%). This was likely due to several factors, for example, poor fixation that resulted in damaged antigen; or inappropriate time for scratching, with the result that monocytes were swept aside. Theoretically, false negative or false positive results should not be found in this immunohistochemical test, since this test used specific antibody and control had also been undertaken to the reagent and non-healthy control-derived serum. For reagent control, the author did not provide primary antibody, and, apparently, the result was negative by the absence of brownish color in the monocyte, indicating a true negative result. Contrastingly, in 7 non-DHF cases, immunohistochemical test using streptavidin biotin revealed positive result in 1 patient (14.2%). This positive result was possibly because in the previous incubation period the dengue virus had invaded the patient's monocyte target cell, but IgM and IgG had not been formed in the serum. This proved that immunohistochemical test provided rapid result to find the presence of dengue virus infection. Current examinations for establishing the diagnosis still require the presence of antibody, so that the results are sometimes negative as it takes time to wait for the emergence of the anti-dengue antibody.

CONCLUSION

The detection of dengue virus antigen in monocyte surface using immunohistochemical examination with streptavidin biotin can be used as diagnostic test for DHF patients and will provide faster positive results than currently available examination method that are based on the emergence of anti-dengue antibody. Immunohistochemical examination with streptavidin biotin is a diagnostic test with a high sensitivity and specificity level (88% and 87%), so that it has a reliable diagnostic value and can be used as DHF diagnostic tool.

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