

RESEARCH ARTICLE

Antibodies in Sera of Dengue Patients with Plasma Leakage Cross-Reacting with DENV Protein and Endothelial Protein

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Abstract

BACKGROUND: Dengue infection remains a major public health problem in Indonesia. Severe dengue associated with plasma leakage require hospitalization and potentially life threatening. However, the mechanism remains unclear, and the occurrence is unpredictable. The role of anti-endothelial antibody is predicted play an important role in the pathogenesis of plasma leakage, as severe dengue is more prevalent in secondary infection or post vaccinated individuals.

METHODS: Serum samples from 127 single Dengue Virus (DENV) serotype infected subjects were obtained in day 2 of fever onset. Subjects were divided into plasma leakage and non-plasma leakage based on World Health Organization (WHO) criteria. Anti-endothelial antibody in patient sera were detected using western blot of Human Umbilical Vein Endothelial Cells (HUVEC). To confirm cross-reactivity, the sera was preabsorb with mix-DENV lysate.

RESULTS: Three prominent bands were identified on western blot strips that inhibited by pre-absorption with DENV lysate. Plasma leakage patient expressed significantly more antibodies, with 51.7% of plasma leakage patients expressed at least two bands out of those three, compared to 18.5% of non-plasma leakage.

CONCLUSION: Antibodies found in sera of dengue patients with plasma leakage cross-reacted with DENV proteins and endothelial proteins 37 kDa, 75 kDa, 120 kDa, and therefore may be involved in the pathogenesis of plasma leakage. Proteomic identification of those protein targets is needed and may be useful for vaccine studies and further development of predictor marker for plasma leakage in dengue.

KEYWORDS: severe dengue, plasma leakage, cross-reactive, anti-endothelial antibody

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Introduction

Dengue infection is an acute systemic viral infection caused by four different serotype of Dengue Virus (DENV), namely DEN-1, DEN-2, DEN-3, and DEN-4, which is transmitted by *Aedes aegypti*. The clinical manifestations ranging from mild non-specific fever to life threatening shock syndrome. World Health Organization (WHO)

recorded increased number of dengue cases including severe cases annually. It was recorded 5.2 million cases in 2019, and the death was increased nearly 4-fold from 2000 to 2015. From 3.9 billion people globally at risk, more than 70% of the actual burden is in Asia.(1,2) In Indonesia, dengue infection remains one of major health problems since 1968. During rainy season the number of cases may steeply increase and potentially outbreak. Based on data of 2006 to 2015, the average number of cases was 612,005

cases annually, with nearly 50% severe cases that needed hospitalization.(3) Before 1995 dengue infections were mostly pediatric cases, but since 1984 the incidence was increased among group ages above 15.(4)

Morbidity and mortality of dengue infection have been associated with shock syndrome due to plasma leakage to extravascular compartment leads to end organs failure and death. Shock syndrome usually occurs unpredictably during defervescence on critical phase after day 3 of onset. (5) Pathophysiologic mechanism of plasma leakage is not completely understood. Host and viral factors may play important roles.

One of the proposed mechanisms is that plasma leakage occurs due to high level of pro-inflammatory cytokine released by the host immune system as response to viral infection, known as cytokine storm, that leads to increase endothelial permeability. Previous study reported that, immune complex of pre-existing antibody enhanced virus uptake by monocyte, and therefore increased the inflammatory response. Besides, viral NS1 protein has a direct damaging effect on endothelial, as reported by previous studies.(6-8) However, it may be other possible mechanisms that play a major role, as plasma leakage occurs during defervescence after viremia and acute phase subsided.

Moreover, as reported before, regardless the DENV serotype dengue infection tends to be more severe in secondary infection and primary infection on post vaccinated individuals. Dengue infection induced synthesis of specific antibodies, among them anti-NS1 and anti-E antibodies known to have role in immunopathogenesis of dengue infection. Anti-NS1 antibody is known to cross react with platelet that leads to platelet activation and hemorrhagic diathesis. On the other hand, anti-E antibody is known to be neutralizing antibody and serotype specific. Therefore, E protein had been used as serotype specific dengue vaccination. However, to other serotypes anti-E antibody is non-neutralizing antibody and may be play a role in antibody enhancement yang that boost immune response in subsequent infection with another serotype.(9-11)

Based on these condition above, we hypothesized that there may be antibodies induced by DENV infection or vaccination that cross-react with endothelial cells and subsequently induced transient endothelial damage that leads to plasma leakage. Therefore, in this study we identified the antibodies in acute phase sera of dengue infected patients that potentially cross-react with components of endothelial cell.

Methods

This prospective study was conducted in Clinical Pathology Department, Faculty of Medicine, Universitas Indonesia, and had been approved by Health Research Ethical Committee, Faculty of Medicine, Universitas Indonesia/ Cipto Mangunkusumo General Hospital, Jakarta (Approval No. 1419/UN2.F1/ETIK/PPM.00.02/2020). One hundred and ninety subjects were recruited into this study, and all of them were hospitalized in a private hospital in Jakarta within first three day of fever onset. Serum samples were collected on day 2 of fever.

Dengue infection was confirmed by Real Time-Polymerase Chain Reaction (RT-PCR) abTest™ DEN 4 qPCR II kit (v2.1) (AITbiotech, Singapore). Subjects with multiple DENV infection, pregnancy, and previously known immunodeficient or hepatic disorders were excluded from this study. All 127 eligible subjects were followed for seven days. Clinical and laboratory data during hospitalization were obtained from medical records. The infection status was determined by detection of Immunoglobulin (Ig)M and IgG anti-dengue antibodies. Patient without IgG anti-dengue detectable were considered as primary infection.

Plasma leakage was defined according to WHO criteria and Dengue score. WHO criteria used any of the following: hemoconcentration with hematocrit increased >15%, hypoalbuminemia marked by decreased of serum albumin level >0.5 mg/dL from baseline, or serum albumin level <3.5 mg/dL, or evidence of pleural effusion or ascites proved by ultrasound.(4) For Dengue Score each of the criteria was scored 1, and total score 0-1 was for mild dengue infection, 2 was for moderate dengue infection, 3-4 was for severe dengue infection. The criteria of Dengue Score included hematocrit >15.1%, serum albumin level <3.49 g/dL, and thrombocyte count <49,500/ μ L.(12)

Human Umbilical Vein Endothelial Cells (HUVEC) Preparation

HUVEC was isolated from umbilical cord vein according to previously published protocol.(13) After rinsing to wash off excess blood including in the umbilical vein lumen by inserting Phosphate Buffered Saline (PBS), the umbilical cord was cut on both ends to make a fresh cut. Firstly, the leaks along the cord were checked and then 10 mL collagenase was then inserted into the umbilical vein lumen and confirmed that filled the entire length of the vein and secured by clamping both end of the umbilical cord. After 30 minutes incubation in room temperature the collagenase

was collected in a conical tube, and 20 mL of Roswell Park Memorial Institute (RPMI) medium + Fetal Bovine Serum (FBS) was injected to rinse, and all solution was collected. After washing procedure, the pellet was resuspended in 1 mL of RPMI + FBS and plate in TC dish to expand, incubated at 37°C overnight, and the medium was refreshed daily until the number of cells was sufficient.

Western Blotting

To detect antibody against endothelial cells, the HUVEC lysate were blotted on to nitrocellulose membrane according to standard procedure of western blot. The lysate was mixed with Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing 5%-mercaptoethanol (Bio-Rad). After separation by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), the protein was transferred to nitrocellulose membrane, and cut into strips. Nitrocellulose membrane strip was blocked with 5% skimmed milk in Tris buffer for 1 hour, followed by adding 40 µL serum samples, incubated overnight (25±3°C) on a rocking platform. After washing procedures, secondary antibody, goat anti-human IgG alkaline phosphatase conjugated was added, and incubated. Following washing procedures, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate was added to develop bands of antibodies. To prove that the antibodies cross-react between endothelial cells and DENV, western blotting were repeated after serum samples pre-absorbed with lysate of mixed DENV1-4. Pre-absorption were done by incubating 5 µL serum sample with 495 µL PBS/0.3% Tween/5% skimmed milk and 2 µg/mL mix DEN lysate. Following 30 minutes incubation in room temperature, the mixture was centrifuged 12,000 rpm for 5 minutes. The supernatant was run for western blot. Any antibodies that were unseen on the WB strip after incubation were considered as cross-reactive antibody.(14)

Statistical Analysis

Data were analyzed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). Normality of numeric data were tested by Kolmogorov-Smirnov. For non-normally distributed data comparison of two means were done with Mann-Whitney test. Chi-square were used to analyze the difference of categorical data.

Results

Out of 190 patients recruited, 69 subjects were excluded from this study due to multiple or undetected DENV

infection. The remaining 121 patients were followed for 7 days until discharge. The demographic and clinical data of the studied group are demonstrated in Table 1. Study subjects were classified into 2 groups based on WHO plasma leakage criteria. The proportion of male subjects was higher in the non-plasma leakage group, while the proportion of female subjects was higher in the plasma leakage group. The patients' median age was 33 years old in non-plasma leakage group and 36 years old in plasma leakage group, with no statistical difference between both groups ($p=0.12$).

The proportion of infecting DENV serotypes were similar in both plasma leakage and non-plasma leakage groups, with DEN-3 being the least infecting serotype. Most patients in the non-plasma leakage group (64.9%) were having primary infection, while in the plasma leakage group only 51.2% were having primary infection. Significant differences were seen in the degree of hemoconcentration represented by increased hematocrit, with a median of 9.0% in non-plasma leakage group, and of 13.3% in plasma leakage group ($p<0.001$). Serum albumin levels were also significantly lower in plasma leakage group (median 2.9 g/dL) compared to non-plasma leakage group (median 3.6 g/dL) ($p<0.001$). All patients with plasma leakage had low thrombocyte counts, with a median of 41.0 (IQR: 24.0-80.0) $\times 10^3$ cells/ μ L and significantly lower ($p<0.001$) from non-plasma leakage group whose median was 107.0 (IQR: 83.5-165.3) $\times 10^3$ cells/ μ L. There were 52 patients with pleural effusion/ascites detected by ultrasound, and all of them belong to plasma leakage group. When the patients were grouped based on dengue score, most patients in non-plasma leakage (89.2%) had dengue score of 0-1, 10.8% scored 2, and no patients scored 3-4. In contrast, in plasma leakage group 54.7% scored 3-4, 25.0% scored 2, and 21.4% scored 0-1.

Antibody reactivities of patients' sera against endothelial protein (anti-endothelial antibody) were exhibited by western-blotting using endothelial cell lysate as targets. Several bands were seen on the strips, but there are three prominent bands disappeared after adsorption with mix-DENV lysate. Those bands were bands of antibodies against endothelial protein sized about 120 kDa, 75 kDa, dan 37 kDa (Figure 1). For practicality reasons, those bands were going to be named based on the sizes in the following part of this paper.

Plasma leakage patients exhibited significantly more bands out of those three bands ($p=0.005$) compare to non-plasma leakage patients (Table 2).

Antibody anti-endothelial 75kDa protein was the most frequently found band, and significantly more frequent

Table 1. Characteristics of subjects.

Characteristics	Non-plasma Leakage (n=37)	Plasma Leakage (n=84)	<i>p</i> -value
Gender			
Male, n (%)	24 (64.9)	40 (47.6)	
Female, n (%)	13 (35.1)	44 (52.4)	
Age (years old), median (IQR)	33 (25–41)	36 (28–51)	0.12
DENV serotype, n (%)			
DENV-1	12 (32.4)	24 (28.5)	0.39
DENV-2	13 (35.1)	23 (27.4)	
DENV-3	2 (5.4)	11 (13.1)	
DENV-4	10 (27.1)	26 (31.0)	
Infection status, n (%)			
Primary	24 (64.9)	43 (51.2)	0.25
Secondary	13 (35.1)	41 (48.8)	
Increased of Ht (%), median (IQR)	9.0 (6.7–12.4)	13.3 (9.7–18.2)	<0.001*
Serum albumin (g/dL)	3.6 (3.4–3.7)	2.9 (2.7–3.2)	<0.001*
Thrombocyte ($10^3/\mu\text{L}$)	107.5 (83.5–165.3)	41.0 (24.0–80.0)	<0.001*
Pleural effusion/ascites, n (%)	0 (0)	52 (58.4)	
Dengue score, n (%)			
Score 0-1	33 (89.2)	18 (21.4)	
Score 2	4 (10.8)	20 (23.8)	
Score 3-4	0 (0.0)	46 (54.8)	

*Tested with Mann-Whitney.

among plasma leakage patients compare to non-plasma leakage patients ($p=0.005$). While anti-37kDa and -120 kDa proteins expression were not significantly different between the groups ($p=0.06$, $p=0.55$, respectively) (Table 3).

Discussion

Antibody was hypothesized to play an important role on vascular endothelial damage that leads to plasma leakage in dengue infection.(15,16) This study was aimed to detect antibodies induced by DENV infection that may cross react with endothelial component and probably related to endothelial damage that leads to plasma leakage.

Subjects recruited into this study were patients who were hospitalized within three days of fever onset. Only single DENV serotype infected patients were included. All 127 patients were followed up to day 7 of hospitalization to detect whether there were signs of plasma leakage during defervescence, and then grouped based upon it. Subject characteristics within both groups were not significantly different on age, proportion of DENV serotype, and infection status. However, there were significantly differences on

degree of hemoconcentration, the lowest serum albumin level, and the lowest thrombocyte count, consistent with previous studies (17-19) that reported in plasma leakage patients there were significantly higher degree of hemoconcentration, and lower level of serum albumin and thrombocyte count. In addition to grouping based on WHO criteria, our subjects were also grouped based on dengue score, as dengue score provides more detailed grouping. As reported in previous study (20), in our study dengue score 3-4 was only found in plasma leakage group, while dengue score 0-1 were predominant among non-plasma leakage group. However, there were 19 subjects with plasma leakage that had dengue score 0-1. These patients were confirmed plasma leakage based on detection of pleural effusion or ascites by ultrasound, one of WHO criteria but not dengue score criteria.

Previous study reported that NS1 protein were expressed by endothelial cell infected by DENV. The protein was subsequently being target of anti-NS1 antibody, forming a NS1-anti-NS1 complex on the surface of endothelial cells that elicited immune effector responses through classical pathway complement activation, and cytolytic process by Natural Killer cells through antibody-dependent cytotoxicity

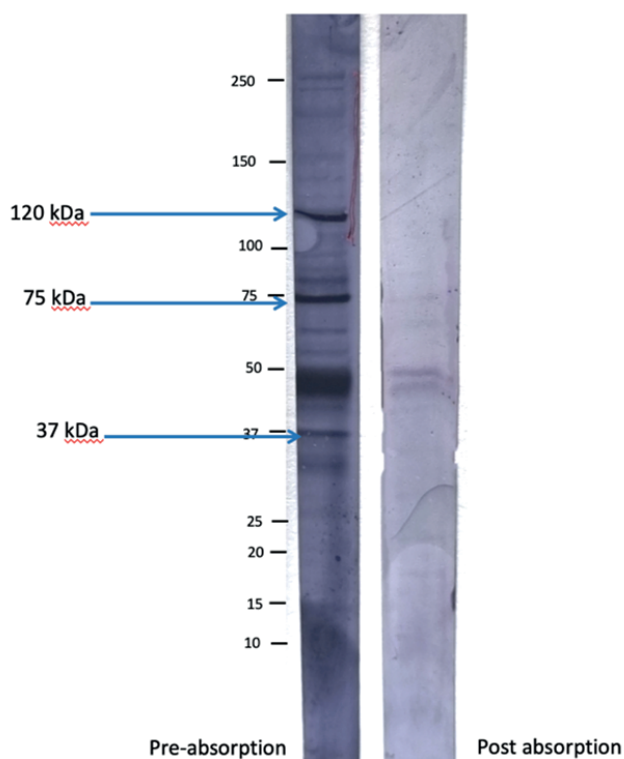


Figure 1. Bands of antibodies of Western-blot strips pre- and post-absorption with mix-DENV lysate.

that might end up with vascular endothelial damage.(21-23) However, as we used uninfected HUVEC in this study, we showed that there were antibodies in DENV infected patient plasma that cross reacted with naïve uninfected endothelial cells. This may be caused by the possibility of molecular mimicry between endothelial cells and DENV proteins. (21,24)

In this study we found three bands that prominently appeared on our western blot strips *i.e.*, antibody against endothelial proteins with predicted sized about 120, 75, and 37 kDa. The size was predicted based on protein marker size, not precisely measured, and we did not further identify

those target proteins yet. Even though there were other bands showed on the western blot strips, the pattern was random, and we were unable to identify the different pattern with non-dengue infected individuals. However, we could not conclude that those bands were not related to dengue infection, as dengue is endemic in Indonesia, most of our population may have been infected and possess antibodies from previous infection.

Although some of patients with plasma leakage did not express any of the antibodies, the expression of multiple antibodies was significantly higher in plasma leakage group, as 52.3% of plasma leakage patients expressed at least two bands out of those three, compared to 18.9% of non-plasma leakage. Based on this finding, we suggest that the antibodies may be associated with plasma leakage in dengue infection. Among those three, anti-75 kDa protein antibody was significantly higher expressed among plasma leakage patients compared to non-plasma leakage. This protein may be attracting for further study and potentially become a marker for plasma leakage predictor.

In concordance with our study, previous study demonstrated that higher percentage cross reactivity of serum samples from Dengue Hemorrhagic Fever (DHF)/ Dengue Shock Syndrome (DSS) patients with HUVEC compared to dengue fever and non-dengue patients. They also showed that DHF/DSS patients sera induced higher endothelial cells apoptosis and lysis. The study was also proved that pretreatment with recombinant NS1 protein of DEN-2 partially prevented such cross-reactivity. Therefore, they suggested that even though anti-NS1 antibody played a significant role in the cross reactivity with endothelial cells, the presence of anti-endothelial antibody not being absorbed by recombinant NS1 could still be detected in patient sera. (25) Moreover, in other study identified an anti-DENV NS1 antibody shared epitope target with a lysine-riche protein (LYRIC) on endothelial cells. LYRIC protein is also called

Table 2. Proportion of anti-endothel antibody among plasma leakage and non-plasma leakage patients.

Anti-endothelial Antibody	n (%)		p-value*
	Non-plasma Leakage (n=37)	Plasma Leakage (n=84)	
No band	10 (27.0)	14 (16.7)	0.005
1 band	20 (54.1)	26 (30.9)	
2 bands	5 (13.5)	31 (36.9)	
3 bands	2 (5.4)	13 (15.5)	

*Tested with Chi-square.

Tabel 3. Proportion of anti-endothelial 37, 75, 120 kDa proteins antibody of plasma leakage and non-plasma leakage groups.

Anti-Endotel Antibody	n (%)		p-value
	Non-Plasma Leakage	Plasma Leakage	
37 kDa	7 (18.9)	31 (36.9)	0.06
75 kDa	14 (37.9)	55(65.5)	0.005
120 kDa	16 (43.2)	42 (50.0)	0.55

*Tested with Chi-square.

metadherin. They suggested that the antibody might lead to transient vascular leakage in DHF/DSS.(24)

As in our study, the antibodies were detected in day 2 sera of dengue infected patients, we were unable to conclude whether the antibodies were synthesized against recent dengue infection, or the infection induced pre-existing autoreactive B lymphocytes to synthesis antibody that cross-react against both endothelial cells and DENV. (26) This study has limitations including the endothelial protein targeted by the antibodies were not yet identified. Besides, we did not include non-DENV infected subjects and patients with multiple DENV serotypes infection. Therefore, further proteomic analysis is needed to confirm proteins/peptides sequences targeted by those antibodies. The strength of our study was the subject were recruited as early as first two day of fever onset and followed along the course of the infection. The dengue infection was confirmed by rt-PCR and only single serotype infected patients were recruited. Furthermore, the detection of plasma leakage aided by ultrasonography that may detect subtle pleural effusion and ascites.

Conclusion

We concluded that there were antibodies in sera of dengue patients with plasma leakage which cross-reacted with DENV proteins and endothelial proteins 37 kDa, 75 kDa, 120 kDa, and therefore, may be involved in the pathogenesis of plasma leakage. Proteomic identification of those protein targets is needed and may be useful for vaccine studies and further development of predictor marker for plasma leakage in dengue.

Authors Contribution

DW, S, and ARH were involved in conceptualizing and planning the research, DW performed the data acquisition/collection,

S and TS provided samples including clinical samples and DENV lysate. A, ARH, and TS involved in planning laboratory tests. SB calculated the experimental data and performed the analysis, DW drafted the manuscript and designed the figures, S and ARH aided in interpreting the results. HTP and IR provided clinical samples. All authors took parts in giving critical revision of the initial study design and manuscript.

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