

The Role of Anti-dengue Virus NS-1 and Anti-protein Disulfide Isomerase Antibodies on Platelet Aggregation in Secondary Dengue Infection

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ABSTRAK

Tujuan: untuk mempelajari korelasi anti-NS-1 dan anti-PDI dengan hambatan fungsi agregasi trombosit pada infeksi dengue sekunder. **Metode:** penelitian ini dilakukan dengan desain potong lintang pada 50 pasien dengue sekunder. Dilakukan pengukuran hambatan fungsi agregasi trombosit serial sampel serum pasien pada hari demam ketiga, lima dan tujuh. Juga dilakukan deteksi anti-NS-1 dan anti-PDI pada sampel yang sama menggunakan metode solid phase ELISA. **Hasil:** terdapat penghambatan agregasi trombosit yang semakin meningkat antar hari pemeriksaan. Terdapat perbedaan bermakna nilai hambatan agregasi antar hari pemeriksaan ($p < 0,05$). Tidak didapatkan penghambatan agregasi trombosit bila dikelompokkan berdasarkan jumlah trombosit $> 100.000/\mu\text{L}$ atau $< 100.000/\mu\text{L}$. Anti-NS-1 dan anti-PDI terdeteksi mulai hari ketiga. Dari 50 pasien didapatkan 90% positif IgG anti-NS-1 dan 72% positif IgG anti-PDI. Kinetika kadar anti NS1 yang dinyatakan dengan OD, tertinggi pada hari ketiga dan mengalami penurunan pada hari ketujuh. Kinetika anti-PDI pada umumnya mengalami peningkatan dari hari ketiga demam sampai hari ketujuh. Terdapat korelasi bermakna ($r = 0,386-0,490$) antara OD anti-NS-1 dan OD anti-PDI, sedangkan perbedaan OD antar hari pemeriksaan tidak bermakna ($p > 0,05$). **Kesimpulan:** anti-NS-1 dan anti-PDI pada infeksi dengue sekunder terdeteksi sejak hari ketiga demam. Terdapat hambatan agregasi trombosit pada pasien dengue sekunder. Anti-NS-1 dan anti-PDI kemungkinan berperan pada hambatan fungsi agregasi trombosit tetapi secara statistik tidak menunjukkan korelasi bermakna. Mungkin ada mekanisme lain yang turut berperan pada hambatan agregasi trombosit.

Kata kunci: anti NS-1, anti PDI, hambatan agregasi trombosit, infeksi dengue sekunder.

ABSTRACT

Aim: to observe the correlation between anti-NS-1 and anti PDI antibodies against platelets function disorder on secondary dengue infection. **Methods:** 50 patients with secondary DV infection according to WHO criteria were observed by a cross sectional study. Patient's blood was collected on day 3, 5 and 7 after fever onset. Platelets aggregation test was done to prove the possibility of platelets dysfunction. Anti-NS-1 and anti-PDI antibodies were determined by solid phase ELISA. **Results:** the inhibition of platelets aggregation was increased among day of observation. Means value of inhibition on day 3 is 46.6%, day 5 is 52.5% and day 7 is 56%. There is a significant difference ($p < 0.05$) of inhibition of platelet aggregation value between days of observation. The antibodies against NS-1 DV and PDI were detected in all 50 sera with the positive rate of 90% develop NS-1 antibodies and 72% of PDI antibodies, on day 3 of symptoms. The highest OD of NS-1 antibodies is detected on the day 3 and decreased on day 7. The OD of PDI antibodies was increased on day 3 and still increasing on day 7. There is a significant correlation between anti NS-1 and PDI antibodies ($r = 0.386-0.490$),

while the differences of OD between observation days are not significant ($p > 0.05$). **Conclusion:** the kinetics profile of NS1 and PDI antibodies responses, which were detected by the third day of symptoms. Dengue patients' sera inhibited platelets aggregation. NS-1 antibodies and PDI antibodies might have a role on the platelets aggregation dysfunction; however, there is no correlation between them. It is possible that other mechanism involve in the inhibition of platelets aggregation.

Key words: anti-NS-1 antibodies, anti PDI antibodies, inhibition platelets aggregation, secondary dengue infection.

INTRODUCTION

Dengue infection is one of the major public health problems in Indonesia.¹ The incidence rate of the disease increased rapidly during the last decades. Dengue virus (DV) consist of four distinct serotypes (DENV 1 to 4).^{2,3} Infection with any one of the serotypes can caused a broad spectrum of manifestations from asymptomatic or mild dengue fever (DF) to dengue shock syndrome (DSS).^{4,5} Hemorrhagic syndrome, a feature of DHF/DSS, is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy and vasculopathy related with dysfunction of platelets and endothelial cells.⁶⁻⁷ However, the mechanisms underlying severe bleeding in dengue DHF are not completely understood.⁸ Recently, Falconar et al. proposed that the dengue virus nonstructural-1 protein (DV NS1) generated antibodies to common epitopes on human blood clotting and integrin/adhesion proteins present on the platelets.⁹

The findings of transient thrombocytopenia associated with the generation of antiplatelet antibodies like anti-NS-1 antibodies in dengue virus infected in mice further support the hypothesis that cross-reactive anti-NS-1 antibodies may play a role in dengue pathogenesis.¹⁰⁻¹² Furthermore, anti-NS-1 mediated platelet aggregation inhibition, is at least in part, attributable to its binding and inhibitory effect on Protein disulfide Isomerase (PDI).¹³ However the mechanism of anti-DV NS-1 mediated inhibition of platelet aggregation remains unclear.¹⁴ In this study, we observed the correlation between anti-NS-1 and anti-PDI antibodies against platelets function disorder on secondary dengue infection.

METHODS

Clinical Samples

The study was held at Biology Molecular Laboratory of Hematology and Medical Oncology

Division of the Department of Internal Medicine of Cipto Mangunkusumo National Central General Hospital and Faculty of Medicine University of Indonesia from March 2011 to April 2012.

Serum samples were obtained from 50 patients that hospitalized at the Cipto Mangunkusumo Hospital and Kramat 128 hospital Jakarta. The criteria of inclusion are as follows: patient with sudden fever at least three days who were classified as having dengue haemorrhagic fever according to the WHO criteria (WHO 1997), anti-NS-1 positive, anti-dengue IgG positive and agree to sign an informed consent form. The exclusion criteria included mix infection with another infection illness, liver function disorder, got steroid treatment and patients disagree to participated in this study.

The serum samples were collected from confirmed cases of dengue virus infected patients on day 3, 5 and 7 after fever ($n=50$, total 150). NS1 detection was measured with the commercially available pan bio dengue early ELISA (Cat No. E-DEN01P) and anti-dengue IgM and IgG antibodies were measured with the commercially available AIM dengue triple line rapid test®.

Antigen and Reagents

Dengue virus NS-1 glycoprotein (ab64456, Abcam, UK). PDI protein (ab31810, Abcam, UK). Horseradish peroxidase (HRP)-conjugated Goat anti human IgG Fc from Jackson ImmunoResearch Lab (West Grove, PA, USA). Non-fat dry milk (NFDM) (Zygm). Substrate 3,3', 5,5'-tetramethylbenzidine (TMB) from invitrogen. Agonist adenosin diphosphat (ADP) from Helena platelet aggregation reagents (Helena laboratories).

Analysis of ADP-stimulated Inhibition Platelet Aggregation

Human whole blood was mixed with 0,11 M sodium citrate (9:1) and centrifuged at 100 x g

for 10 min at room temperature. The upper layer was collected as platelet rich plasma (PRP). The lower layer was subsequently centrifuged at 1000 x g for 10 min and the supernatant was collected as platelet poor plasma (PPP). The PPP was used as blank for analysis of platelet aggregation. The PRP was incubated with 5 μ L of DF/DHF patient sera mixture at 37°C for 30 min, followed by addition of ADP (10 μ M final concentration). Inhibition platelet aggregation was detected using an automated aggregometer (Helena AggRam™).

Determination of Anti-NS-1 Antibodies and Anti-PDI Antibodies by Solid Phase ELISA

According against NS-1 antigen in patient sera was analyzed by the solid phase ELISA, according to Alcon et al⁸, Shu et al⁹ and Cheng et al.⁶ Microtiter wells (NUNC, Denmark) were coated with 50 μ L of recombinant Dengue Virus NS1 glycoprotein (5 μ g/mL) or 50 μ L PDI protein (5 μ g/mL) in 0,1 M bicarbonate buffer pH 9,5, incubate 4°C overnight. After blocking with 5% skim in phosphate-buffered saline (PBS), diluted serum sample (1:50 in blocking buffer) was added and incubated overnight at 4°C. Wells were washed 5 times with 200 μ L PBS 0.1% Tween 20 (PBST). 100 μ L of horseradish peroxidase labeled goat anti-human IgG (1:15.000 dilution) was added for 1 h at 37°C. The preparations were then washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB). The enzyme activity was developed; absorbance was measured using infinite f 200 TECAN Reader (Austria) at 450 nm. Serum samples from healthy donors were used as control negative (n=5). Positivity was determined by comparison to individual negative controls. A positive sample was defined as having a test absorbance/negative control ratio of ≥ 2.0 and a negative sample was defined as having a ratio of ≤ 2.0 .

Statistical Analysis

We used the Pearson correlation test to examine correlation between anti-NS-1 and anti-PDI with platelet aggregation. $p < 0.05$ was considered significant. The related ANOVA for comparison between normally distributed OD anti NS1, OD anti-PDI and platelet aggregation and Friedman test was used if data are not normally distributed. For all statistical analysis, we used the SPSS version 17.0 software package.

RESULTS

In this study we analyzed 150 serum samples from 50 patients with laboratory confirmation of inhibition platelet aggregation, IgG NS1 antibodies and IgG PDI antibodies. All of these patients were classified as secondary infection based on interpretative criteria described above.

Dengue Patient Sera Inhibit ADP-induced Platelet Aggregation

The platelet functional assay was also carried out and results showed that the patient serum caused an inhibition on platelet aggregation induced by ADP (**Figure 1**). The inhibitions of platelets aggregation were increased among day of observation. Means value of inhibition on day 3 is 46.6%, day 5 is 52.2% and day 7 is 56%. There is a significant difference ($p < 0.05$) of inhibition of platelet aggregation value between days of observation (Anti-NS-1 antibodies and anti-PDI antibodies in dengue virus infected patients).

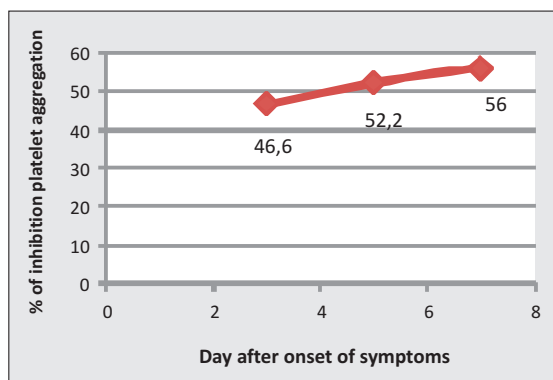


Figure 1. Kinetics of inhibition platelet aggregation in DV secondary infection

The presence of anti NS1 antibodies and anti PDI antibodies in the serum of patients with secondary infection was analyzed using solid phase ELISA. The antibodies against NS-1 DV and PDI were detected in all 50 patients with the positive rate of 90% develop NS-1 antibodies and 72% of PDI antibodies, on day 3 of symptoms. The highest OD of NS-1 antibodies is detected on the day 3 and decreased on day 7. The OD of PDI antibodies was increased on day 3 and still increasing on day 7. (**Figure 2**)

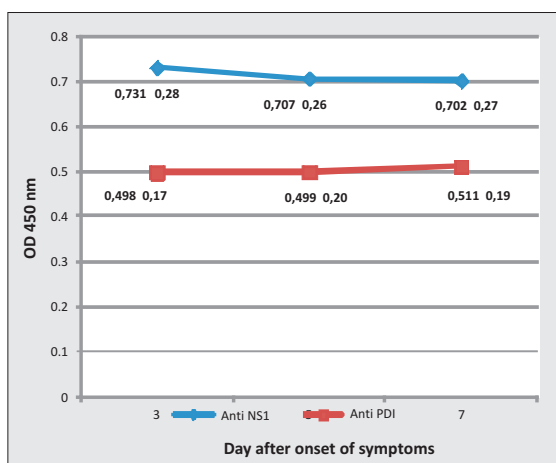


Figure 2. Kinetics of IgG NS1 antibodies and IgG PDI antibodies in DV secondary infection

There is a weak correlation between anti NS-1 antibodies and the inhibition of platelets aggregation ($r=0.160$, $p=0.267$), and also between anti PDI antibodies and the inhibition of platelets aggregation ($r=-0.081$, $p=0.578$).

There is a significant correlation between anti-NS-1 and PDI antibodies ($r=0.386-0.490$, $p<0.01$), while the differences of OD between observation days are not significant ($p>0.05$).

Figure 3.

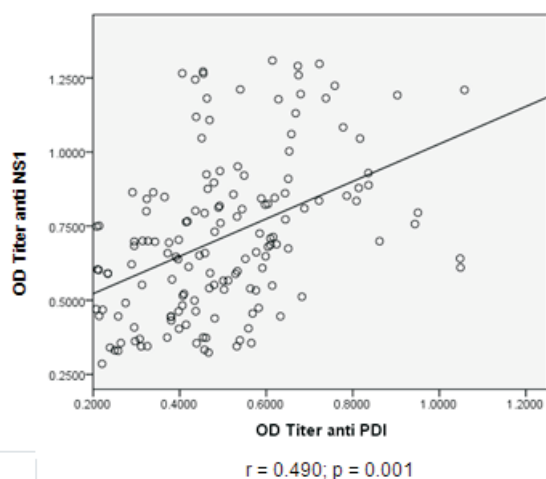


Figure 3. The correlation between anti-PDI antibodies and anti-NS-1 antibodies in day 3, 5 and 7 fever ($r=0,490$; $p=0,001$)

DISCUSSION

DHF is the main cause mortality in dengue virus infection. Hemorrhagic syndrome, a feature of DHF is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy and vasculopathy related with destruction/dysfunction

of platelet and endothelial cells.¹⁰ Although the exact pathomechanism is not very well defined, available data strongly suggest that in the most cases of DHF immune mediated mechanism play also an important role in the destruction of platelets and disturbance of endothelial function.²

Several evidences indicated a mechanism of molecular mimicry in which antibodies against non structural protein NS1 of DENV (anti-NS-1 antibodies) can also cross react with platelet and endothelial cells, thereby may induce platelet destruction and endothelial disturbance in DHF patients.¹⁰

Recent study reported by Cheng et al. demonstrated that anti-NS-1 antibodies recognized PDI on platelet surface causing inhibition of platelet aggregation induced by ADP.⁶ Further analysis showed that anti NS1 antibodies bound to amino acid residues 311-330 of DENV NS1, which shares sequence homology with the thioredoxin domain of PDI.⁶ Interestingly, PDI has been to play a role on the regulation of integrin activation showed that modulation of the thiol isomerase activity of PDI by divalent manganese cation leads to PDI.⁷

In this study, the presence of antibodies directed against platelets was demonstrated in dengue patient sera, which revealed the inhibitory effect on platelet aggregation by DF/DHF patient sera.

CONCLUSION

This study shows the kinetics profile of NS-1 and PDI antibodies responses, which were detected by the third day of symptoms. Dengue patient's sera also inhibited platelets aggregation. NS-1 antibodies and PDI antibodies might have a role on the platelets aggregation dysfunction, but statistically there is no correlation between them. It is possible that other mechanism involve in the inhibition of platelets aggregation.

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