The Relation Between Levels of TNF -Alpha, IL-1β, PGE$_2$ and PLA$_2$ with the Severity Degree of Dengue Hemorrhagic

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The pathogenesis of dengue virus infection is still being debated. Based on the existing data, there is a strong evidence that the immunopathological mechanism plays a role in dengue virus infection with various complications. Some unknown immune responses play a role in the pathogenesis of dengue virus infection. Researchers are trying to establish the role of several inflammatory mediators such as PLA$_2$, IL-1 , TNF- , PGE$_2$, PGI2, Thromboxane A$_2$, Leucotrien and MPTP, in relation to the severity degree of the dengue virus infection. The aim of this study is to recognize the relation between the severity degrees of dengue hemorrhagic fever (DHF) patients and the immunological profile in the sub-cellular level, such as PLA$_2$, IL-1 , TNF- , PGE$_2$ and PLA 2. The collected data was processed and presented analytically. The relation between each parameter (TNFα, SPLA$_2$, PGE$_2$,IL-1β) and the degree of DHF was analyzes, using Spearman's correlation analysis, ordinal regression. It was shown that there was no relation between the levels of TNFα, PGE2, IL-1β, and SPLA2 in patients with various degrees of DHF, but there were significant differences between DHF grade 1 and 3, and also 2 and 3, on IL-1β. There were increased levels of the four parameters in dengue grade 1 to 2, but decreased levels in grade 3. This can be caused by inflammatory processes, but the severity degree of DHF can also be influenced by complement, thromboxane, and leukotrien.

Key words: dengue hemorrhagic fever, cytokine, PLA$_2$, IL-1β, TNF-α, PGE

Patogenesis terjadinya infeksi virus dengue hingga saat ini masih diperdebatkan. Terdapat bukti yang kuat bahwa mekanisme imunopatologi berperan dalam terjadinya infeksi virus dengue dengan berbagai komplikasi. Beberapa penelitian mencoba membuktikan peran beberapa mediator inflamasi seperti sPLA$_2$, IL-1, TNF-, PGE$_2$, Thromboxane A$_2$, Leucotrien, dan MPTP dalam hubungannya dengan derajat keparahan dari infeksi virus dengue tersebut. Penelitian observasional ini dilakukan dengan rancang bangun cross sectional. Terdapat 45 penderita yang dirawat di Ruang Penyakit Infeksi RSUD Dr. Soetomo yang memenuhi persyaratan. Berdasarkan pemeriksaan IL-1β (correlate enzyme assay), TNF-α, PGE$_2$ dan sPLA$_2$ dengan metode EIA diketahui tidak didapatkan korelasi yang bermakna antara pengaruh IL-1β, TNF-α, PGE$_2$ dan sPLA$_2$ terhadap derajat keparahan deman berdarah dengue (DBD). Terdapat peningkatan kadar pada DBD derajat 2 dibandingkan dengan derajat 1, tetapi pada derajat 3 terdapat penurunan kadar dari semua parameter. Perbedaan yang bermakna ditemukan hanya pada pengaruh IL-1β terhadap derajat keparahan DBD. Perbedaan bermakna didapatkan antara DBD derajat 1 dan 3 serta antara DBD derajat 2 dan 3. Tidak didapatkan hubungan linear antara IL-1β, TNF-α, PGE$_2$, dan sPLA$_2$ dengan derajat keparahan DBD, tetapi pada IL-1β terdapat perbedaan bermakna antara derajat 1 dengan 3, serta derajat 2 dengan 3. Hal ini mungkin disebabkan penurunan proses inflamasi pada derajat 3, tetapi beratnya derajat DBD mungkin dapat dipengaruhi oleh komplemen, tromboksan, leukotrien, atau faktor lain yang mempengaruhi permiabilitas endotel dan kerusakan endotel kapiler.

Kata kunci: demam berdarah dengue, sitokinin, PLA$_2$, IL-1β, TNF-α, PGE

Dengue hemorrhagic fever (DHF) is one the health problems in the tropical region. In Southeast Asia, with a total population of 1.5 billion, nearly 1.3 billion people are at risk of dengue virus infection. Dengue is a major cause of hospitalization and death in children. DHF was first reported in Indonesia in 1968, namely Jakarta and Surabaya. The DHF incidence rate continues to increase (Nasronudin 2007).

Dengue virus infection is actually a self-limiting disease, but is often accompanied by dangerous complications, such as plasma fluid leakage characterized with haemo-concentrations (increased hematocrytes), shock and bleeding (WHO 2008; Garcia et al. 2010).

The pathogenesis of dengue virus infection is still being debated. Based on the existing data, there is strong evidence that the immunopathological mechanism plays a role in dengue virus infection with various complications as mentioned above. Some immune responses are known to play a role in the pathogenesis of dengue virus infection. Researchers are trying to establish the role of several inflammatory mediators, such as PLA$_2$, IL-1 , TNF- , and PGE2, in

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relation to the degree of severity of the dengue virus infection. So far, not many researchers have been interested in studying the above mentioned inflammatory mediators (Khrisnan *et al.* 2007; Miller *et al.* 2008).

MATERIALS AND METHODS

Sample Preparation. The analytic study in this research was an observational study with cross-sectional design. The research was conducted from February to November 2009 at the Infectious Diseases ward of Dr. Soetomo Hospital: selection and blood sampling, Institute of Tropical Disease, Airlangga University, the Department/Installation of Clinical Pathology Airlangga University School of Medicine, Dr. Soetomo Hospital: plasma separation, platelet tests, hematocryte, dengue IgG and IgM, NS1, plasma sPLA2 activity, Prostaglandins 2 plasma, IL-1β, TNF-α.

Samples were taken from 45 hospitalized patients at the Tropical Infectious Diseases ward at Dr. Soetomo Hospital, Surabaya, with DHF as a diagnosis based on WHO criteria in 1997, and positive results of serological tests and/or IgG-anti-dengue IgM and/or NS-1 antigen, meeting the criteria for inclusion and exclusion.

Inclusion Criteria. (i) Based on the 1997 WHO criteria, diagnosis of DHF is established when all of the following components are met: fever or history of acute fever, between 2-7 days, usually biphasic, at least one of bleeding manifestations, such as (positive Rumple-Leede test), ptechiae, ecchymosis, or purpura, mucosal bleeding (epistaxis or gum bleeding), or bleeding from other sites (haematemesis or melena), thrombocytopenia (platelets <100 000 μL^{-1}), at least one of plasma leakage signs (such as increased hematocryte > 20% compared to standard according to age and sex, decrease in hematocryte > 20% after fluid replacement therapy compared with the previous value, other plasma leakage signs such as pleural effusion, ascites or hypoproteinemia). (ii) With positive result of IgG anti-dengue, and/or IgM anti-dengue and/or NS-1 (Non-structural-1) antigen DHF patient (McBride 2009; Thomas *et al.* 2010). (iii) Age ≥ 14 years. (iv) Willing to participate in this study and signed an informed consent. Patients younger than 21 years can be represented by their parents in signing the informed consent.

Exclusion Criteria. Patients with 2-7 days of fever caused by other than dengue infection, the presence of severe primary diseases (hematological disorders, diabetes mellitus, chronic kidney failure, heart failure, liver failure, liver cirrhosis), and age <14 years.

Studied Variables. Independent variables were levels of necrosing tumor factor-α, interleukin-1 β, secretory phospholipase-A2 plasma, and prostaglandin-2; and dependent variables were severity degrees of dengue hemorrhagic fever. Quality assurance examination of TNFα, SPLA2, PGE2, IL-1β with precision control, which seeks imprecision of the examined sample duplicate (within run). Control accuracy was not done due to lack of accuracy control materials.

Data Analysis. The collected data will be processed and presented analytically: The relation of each parameter (TNFα, SPLA2, PGE2, IL-1β) with the degree of DHF, using Spearman's correlation analysis, the relation of four parameters with the degree of DHF, using multiple ordinal regress enzyme immunometric assay.

Enzyme Immunometric Assay for TNFα, SPLA2, PGE2, IL-1β. After pipetting 100 μL of standard diluents into S0 (0 pg mL^{-1}) standard wells, and pipetting 100 μL of standard 1 through 7 into appropriate wells, take 100 μL of samples into appropriate wells and tap the plate gently to mix the contents. Seal the plate and incubate at 37 °C for 2 h. After medium discharge, empty the contents of the wells and wash by adding 400 μL of wash solution to every well, then repeat the wash 3 more times for a total of 4 washes. After the final wash, remove or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. Then pipette 100 μL of yellow antibody into each well, except the blank then seal the plate and incubate at 37 °C for 1 h. Remove again the content of the wells and wash by adding 400 μL of wash solution to every well and add 100 μL of blue conjugate to each well, except the blank and seal the plate and incubate at 37 °C for 30 min. Finally, empty the content of the wells and wash by adding 400 μL of wash solution to every well and pipette 100 μL of substrate solution into each well after 30 min for incubation at room temperature. Take and pipette 100 μL of stop solution 2 to each well. Blank the plate reader against the blank wells, read the optical density at 450 nm, preferably with correction between 570 nm and 590 nm. If the plate reader cannot be blanked against the blank wells, manually substrate the mean optical density of the blank wells from all the readings relation of four parameters with the degree of DHF (not linear), using anova.

RESULTS

Characteristic of Subjects. This research is an observational analytic study with a case-control design

in order to determine the relation between TNF-α levels, IL-1β, PGE2, and PLA2 and the severity degree of DHF. Research subjects were DHF patients who met the criteria for the sample acceptance. Samples were taken at the Tropical Disease ward, Dr. Soetomo Hospital, Surabaya, from March to September 2009. During the collection of samples until the examination of levels of TNF-α, IL-1β, PGE2, and PLA A-2; the plasma was stored in the Tissue Bank at Dr. Soetomo Hospital, Surabaya, and the Institute of Tropical Diseases, Airlangga University. A -70 °C storage was needed in order to maintain bioactivity.

Based on the results, there were 45 DHF patients with the following details: 7 patients with DHF grade 1 (15.6%), 35 patients with DHF grade 2 (77.8%) and 3 patients with DHF grade 3 (6.7%). DHF grade 4 was not found in this study. Most of the cases were DHF grade 2.

Of the 45 patients, 31 were male (68.9%) and 14 were female (31.1%), with an age range of 14 to 48 years old, and the mean was 21 years old. The levels of TNF-α were 73.2 to 809.7 pg mL^{-1}, with a mean of 199.7 pg mL^{-1}; the levels of IL-1 β were 12.7 to 45.9 pg mL^{-1}, with a mean of 31.6 pg mL^{-1}; the levels of PGE2 were 39.1 to 1350 pg mL^{-1}, with a mean of 238.09 pg mL^{-1}, and the levels of PLA A-2 were 37.8 to 195.9 U mL^{-1}, with a mean of 97.48 U mL^{-1}. The characteristics of samples can be seen in Table 1.

Quality Assurance. Quality assurance of levels of TNFα, sPLA2, PGE2, IL-1β was performed by seeking imprecision within run absorbance. Determination imprecision was performed on different patient samples by double or duplicate examinations, and the result can be seen in in Table 2.

Table 1 Characteristics of research subjects

Characteristics	Mean	SD
Age (y.o.)	21.3600	6.2050
TNF-α (pg mL^{-1})	199.7378	143.63442
IL-1β (pg mL^{-1})	31.6333	6.45720
PGE$_2$ (pg mL^{-1})	238.0911	207.44473
PLA A-2 (U mL^{-1})	97.4933	30.06433

Table 2 Imprecision within run absorbance TNFα, sPLA$_2$, PGE$_2$,IL-1β

Parameter	N	SD	CV (%)
TNFα	10	0.00732	2.00
SPLA2	10	0.01570	2.92
PGE2	5	1.34000	4.97
IL-1β	10	0.000803	2.1

The research findings will be meaningless if the data is not valid. In this study, in order to indicate the data was performed with valid quality assurance, the examination of the levels of TNFα, sPLA2,PGE2, IL-1β   was performed with precision control to measure imprecision.

Relation Between the Levels of TNFα with the Severity Degree of DHF. In this study, the linear correlation between TNFα levels and the severity degree of DHF was not found, with no significant difference (p = 0.922). It was discovered in DHF grade 1 that the mean level of TNFα was 139.9571 pg mL^{-1}, DHF grade 2 was 216.4629 pg mL^{-1} and grade 3 was 144.1000 pg mL^{-1} There were elevated mean levels in DHF grade 1 and 2, but decreased levels in DHF grade 3.

Relation Between the Levels of sPLA$_2$ with the Severity Degree of DHF. In this study, the sPLA2 levels were not linearly correlated with the severity degree of DHF, with no significant difference (p = 0.709). It was discovered in DHF grade 1 that the sPLA2 mean level was 87.5429 U mL^{-1}, DHF grade 2 was 100.3486 U mL^{-1}, and DHF grade 3 was 87.4000 U mL^{-1}. There were elevated levels of sPLA2 in DHF grade 1 and 2, but decreased levels in grade 3.

Relation Between Levels of PGE2 and the Severity Degree of DHF. In this study, the PGE2 levels were not linearly correlated with the severity degree of DHF, with no significant difference (p = 0.929).   It was discovered in DHF grade 1 that the PGE2 mean level was 175.8571 pg mL^{-1}, DHF grade 2 was 258.5171 pg mL^{-1}, and 3 DHF grade 3 was 145.0000 pg mL^{-1}. There were elevated mean levels of PGE2 in DHF grade 1 and 2, but decreased levels in grade 3.

Relation Between Levels of IL-1β and the Severity Degree of DHF. In this study, IL-1β levels were not linearly correlated with the severity degree of DHF, with no sigificant difference (p = 0.929). It was discovered in DHF grade 1 that the IL-1β mean level was 28.9571 pg mL^{-1}, DHF grade 2 was 33.2143 pg mL^{1}, and grade 3 was 19.4333 pg mL^{-1}. There were elevated levels of IL-1β in DHF grade 1 and 2, but decreased levels in grade 3.

This study found a significant difference in IL-1β (p = 0.000) against the severity degree of dengue by using anova. Significant differences were found between DHF grade 1 and 3 with a mean difference of 9.5238, with a standard error of 3.7653 (p = 0.040); between DHF grade 2 and 3 with a mean difference of 13,7809, with a standard error of 3,2825 (p = 0.000). Linear correlation was not found.

Relation between levels of IL-1β, TNF-α, PGE2 and PLA2 with the severity degree of DHF. The influence of IL-1β, TNF-α, and PLA2 on the severity degree of DHF was not found significant, with each p = 0.402 p = 0.589 p = 0.959, as well as the influence

of IL-1β, TNF-α, and PGE2 on the severity degree of DHF, was not found significant with each $p = 0.332$, $p = 0.594$, $p = 0.696$.

DISCUSSION

Imprecision of TNFα, sPLA2, PGE2, IL-1β was below 5%. Imprecision is a deviation of the results compared to the average values, the lower the deviation (which is determined by a standard deviation or coefficient of variation), the more similar result from a series of examinations. An acceptable deviation for particular examination parameters was stated as the coefficient of variation. Generally, the coefficient of variation should not exceed 5%, except for certain parameters which are allowed up to 10%.

In this study, the linear correlation between TNFα levels and the severity degree of DHF was not found. The condition was due to a decrease of inflammatory processes in DHF grade 3, and the severity degree of DHF may be influenced by complement, thromboxane, leucotrien or other factors that affected endothelial permeability and capillary endothelial damage, resulting in more severe plasma leakage (Gulati and Maheshwari. 2007; Whitehorn and Farrar 2010). The sPLA2 levels were not linearly correlated with the severity degree of DHF. The condition was due to a decrease of inflammatory processes in DHF grade 3, and the severity degree of DHF may be influenced by complement, thromboxane, leukotrien or other factors that affect endothelial permeability and capillary endothelial damage, resulting in more severe plasma leakage. The situation can also be caused by a specific immune response to viral infection which produced TNFα as a result of stimulation in B lymphocytes. B lymphocytes respond to DENV-3 antigen, DENV antigen-1, DENV-2, and DENV-4 even though in lower levels. This allows a cross response between each different serotype, which makes a variation of responses to the activation of T lymphocytes (Chaturvedi *et al.* 2000).

The PGE2 levels were not linearly correlated with the severity degree of DHF. This condition can be attributed to the decrease of inflammatory processes in DHF grade 3, and the severity degree of DHF may be influenced by complement, thromboxane, leukotrien or other factors that affect endothelial permeability and capillary endothelial damage, resulting in more severe plasma leakage. PGE2 was affected by sPLA2 and this study showed the same pattern of increase and decrease of these parameters on the severity degree of DHF. It is concluded that there was a sPLA2 influence on PGE2. Although the level of PGE2 not linearly correlated with the severity degree of DHF, but there were found

decreasing increasing level from DHF grade I to grade 2, but the level decrease in grade 3 that maybe due to inflammatory decreasing in grade 3. This study found a significant difference in IL-1β ($p = 0.000$) against the severity degree of dengue by using anova. The reason of that is the same above.

Based on the data above, there was no linear correlation between these parameters and the severity degree of dengue, even though there were increased levels in grade 1 and 2, but then decreased in grade 3. This study found a significant difference only in IL-1β, ($p = 0.000$) against the severity degree of DHF by using anova. Significant differences between DHF grade 1 and 3 were found with a mean difference of 9.5238, with a standard error of 3.7653 ($p = 0.040$), between dengue 2 and 3 degrees with a mean difference of 13.7809, with a standard error of 3.2825 ($p = 0.000$). There was not any linear relation.

The condition was due to a decrease of inflammatory processes, but the severity degree of DHF can be influenced by complement, thromboxane, leukotrien or other factors that affect endothelial permeability and capillary endothelial damage, resulting in more severe plasma leakage. There were similar patterns against the severity degree of DHF on four parameters above, which were higher in grade 2 compared to grade 1, and lower in grade 3.

There were elevated levels of the four parameters in DHF grade 1 to 2, but decreased levels in grade 3. The condition was due to a decrease of inflammatory processes, but the severity degree of DHF can be influenced by complement, thromboxane, and leukotrien.

In this study, the cytokine level (TNFα, SPLA2, PGE2, IL-1β) elevated in DHF garde 1 to grade 2, but that parameters decreased in grade 3. This condition may due to a decrease of inflammatory processes in DHF grade 3. So in this study increasing the cytokines level (TNFα, SPLA2, PGE2, IL-1β) not linier with severity degree of DHF, its may due to decreasing inflammatory in grade 3.

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