

## Erythropoiesis Differences in Various Clinical Phases of Dengue Fever using Immature Reticulocyte Fraction Parameter

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### Abstract

**Objective:** To determine the mechanism of erythropoiesis that led to anemia using the Immature Reticulocyte Fraction (IRF) parameter in various clinical phases of dengue fever.

**Methods:** This study was a comparative analytical research using secondary data derived from the *Dengue-associated Endothelial Cell Dysfunction and Thrombocyte Activation* (DECENT) research. The study was performed at Dr. Hasan Sadikin Hospital Bandung, Indonesia from March 2011 to March 2012. Patients were grouped into fever, critical, recovery, and convalescent phases and a healthy control was established. Data collected were analyzed using the Kolmogorov-Smirnov normality test, followed by Friedman test and Mann-Whitney post hoc test.

**Results:** There were 244 subjects participating in this study. The median IRF for all subjects was 4.8% with an IQR of 2.4-8.1%. The values of Immature Reticulocyte Fraction in fever-phase, critical-phase, recovery-phase, convalescent-phase and healthy-control were 1.8% (IQR of 0.5–2.85%), 3.6% (IQR of 1.8–5.0%), 7.05% (IQR of 4.08–11.85%), 7.3% (IQR of 3.95–9.3%) and 4.1% (IQR of 2.2-6.6%), respectively. There was a significant difference in IRF between groups ( $p < 0.05$ ). The immature Reticulocyte Fraction in fever phase was significantly different from the IRF in other phases and healthy controls ( $p < 0.05$ ).

**Conclusions:** There are changes in erythropoiesis activities detected through the IRF in various clinical phases of dengue infection. Erythropoiesis suppression occurs mainly during the fever phase and starts to be restored in the critical phase. In the recovery and convalescent phases, the erythropoiesis activities increase. This is the first study describing IRF in multiple phases of dengue disease.

**Keywords:** Diastolic dysfunction, hypertensive heart disease, left ventricular hypertrophy, matrix metalloproteinase-9

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### Introduction

Dengue infection is the most notable viral infection transmitted by mosquitoes, seen from the medical perspective and also the

community health perspective. The incidence of dengue infection has increased significantly every year, from 0.05/100.000 in 1968 to 35-40/100.000 in 2013.<sup>1</sup> Indonesia is a dengue virus (DENV) endemic region and has experienced a 700-fold increase in incidence over the past 45 years.<sup>2</sup>

The hematological disorders known in dengue viral infection are temporary thrombocytopenia and leucopenia. The mechanism of neutropenia during dengue infection could be caused by bone marrow

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suppression or peripheral destruction. Changes in erythropoiesis is also observed in dengue infection, which may be caused by the bone marrow suppression affecting all of the hematopoiesis series. It is hypothesized that a combination of viral infection on hematopoietic progenitor cells, and the viral infection on bone marrow stromal cells and dengue specific T-cell activation, both releasing cytokines that suppress the bone marrow.<sup>3,4</sup>

Erythropoiesis suppression during dengue infection was also shown in the form of aplastic anemia by several case reports. Khoj et al reported aplastic anemia occurred after dengue infection and ended with bone marrow transplantation.<sup>5</sup> A study by Lora *et al.*<sup>6</sup> in Dominican Republic indicated that anemia related to the severity and mortality of dengue infection, found in 19% of patients and 32% of severe dengue infection with an Odd Ratio of 3 for mortality. Clinical manifestation of dengue infection in thalassemic patients was different, where they experienced the decrease of hemoglobin rather than hemoconcentration.<sup>7</sup>

Changes in erythropoiesis in dengue infection has not been extensively studied. Bone marrow examination is an invasive procedure and not recommended in dengue infection. A simple hematological parameter such as Immature reticulocyte fraction is expected to be able to indicate how active the erythropoiesis in the bone marrow is.<sup>8</sup>

Immature reticulocyte fraction (IRF) is a parameter reflecting the most immature reticulocyte fraction. This IRF parameter is simple and can be obtained directly from the automated hematology analyzer Sysmex XE-2100 (Kobe, Japan). The IRF reflects erythropoiesis directly and identifies erythropoiesis earlier than reticulocyte and hemoglobin. A study from Goncalo in hematological malignancy patients undergoing hematopoietic progenitor cells transplantation showed IRF as an earlier indicator for success than neutrophil and thrombocyte with two to four days of difference.<sup>8-10</sup> This study is aimed to order to observe whether erythropoiesis is really suppressed during Dengue infection.

## Methods

This is a cohort study, using data derived from the Dengue-associated Endothelial Cell Dysfunction and Thrombocyte Activation (DECENT) research in the Department of Internal Medicine which ran from March 2011 to March 2012. The DECENT study was

designed as a cohort study that recruited patients presenting with clinical signs of symptomatic Dengue virus infection (SDVI) to Dr. Hasan Sadikin General Hospital in Bandung. Consecutive patients meeting inclusion criteria were enrolled and followed with daily clinical assessment, blood collection and, in a subgroup; assessment of plasma leakage until day 5 post-admission. Temporal changes on laboratory (thrombocytes and endothelial cells) parameters and plasma leakage during the infection were determined. Patients were asked to return for follow-up blood collection at >2 weeks (14–20 days) after discharge. Inclusion criteria are subject must be 14 years old or above, and clinical suspicion or confirmation of having DF or DHF/DSS according to WHO criteria. Exclusion criteria are pregnancy, clinical symptoms/signs of or known malignancy, known coagulation disorder, and any chronic diseases such as diabetes mellitus, chronic renal failure, hepatitis, auto-immune disorders, underlying hematological disease, using drugs causing myelosuppression and psychiatric disorders. Health Research Ethics Committee of the Dr. Hasan Sadikin General Hospital Bandung, Indonesia approved all legal and ethical aspects of the study (LB.04.01/ADS/EC/551/XII/2014).

The statistical test using the Friedmann test was used to examine the difference between the phases in dengue patients (4 groups), followed by a *post hoc* Mann Whitney test, the result is considered to be significantly different if the p value is <0.05.<sup>11</sup>

## Results

There were 244 research subjects with a similar ratio of male and female, 56.6% of males and 43.4% of females, with the median age of 24 and IQR (interquartile range) of 14–67. The hemoglobin and hematocrit levels were normally distributed in every phase with a mean of  $14.0 \pm 1.9$  g/dL for hemoglobin and  $41.1 \pm 5$  % for hematocrit. Whereas, the leucocyte and thrombocyte levels were not normally distributed, with the leucocyte median of  $5.100/\text{mm}^3$  and IQR of  $3.800/\text{mm}^3$  to  $6.600/\text{mm}^3$ , and the thrombocyte median of  $78.000/\text{mm}^3$  and IQR of  $41.000/\text{mm}^3$  to  $177.000/\text{mm}^3$ . The baseline characteristics for each phase and control group (Table 1).

The IRF differences in various clinical phases of dengue infection and control group can be seen in Table 2 below. The median IRF for all the research subjects was 4.8% with

**Table 1 Subjects Characteristics**

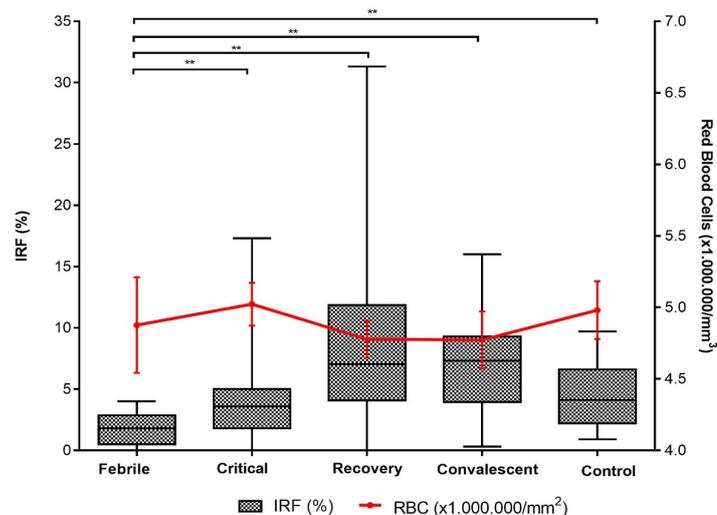
| Variable                                    | Clinical Phase of Dengue Infection |                       |                       |                           | Healthy Control (n=27) | p* value            |
|---------------------------------------------|------------------------------------|-----------------------|-----------------------|---------------------------|------------------------|---------------------|
|                                             | Fever Phase (n=13)                 | Critical Phase (n=77) | Recovery Phase (n=74) | Convalescent Phase (n=53) |                        |                     |
| Sex (n, %)                                  |                                    |                       |                       |                           |                        |                     |
| Male                                        | 8 (61.5)                           | 46 (59.7)             | 42 (56.8)             | 32 (60.4)                 | 10 (37.0)              | 0.292 <sup>c</sup>  |
| Female                                      | 5 (38.5)                           | 31 (40.3)             | 32 (43.2)             | 21 (39.6)                 | 17 (63.0)              |                     |
| Age (year) median                           | 24                                 | 24                    | 23                    | 25                        | 25                     | 0.810 <sup>b</sup>  |
| IQR                                         | (20–28)                            | (19–33)               | (19–33)               | (20–34)                   | (22–31)                |                     |
| Hb (g/dL) mean±SD                           | 13.8±2.4                           | 14.5±2.1              | 13.7±1.7              | 13.9±1.7                  | 14.2±1.8               | 0.076 <sup>a</sup>  |
| Ht (%) mean±SD                              | 40.0±6.1                           | 42.2±5.8              | 39.9±4.8              | 41.5±4.3                  | 42.3±4.7               | 0.040 <sup>a</sup>  |
| Leucocyte (x1000/mm <sup>3</sup> ) median   | 3.3                                | 4.0                   | 5.0                   | 7.1                       | 7.0                    | <0.001 <sup>b</sup> |
| IQR                                         | (2.4–4.3)                          | (3.0–5.6)             | (4.0–6.0)             | (5.7–8.8)                 | (6.5–8.0)              |                     |
| Thrombocyte (x1000/mm <sup>3</sup> ) median | 66                                 | 36                    | 94                    | 265                       | 293                    | <0.001 <sup>b</sup> |
| IQR                                         | (43–89)                            | (19–55)               | (57–132)              | (225–313)                 | (250–318)              |                     |

\*Note : analysis using a: ANOVA test, b: Friedman test, c: Chi Square test, significant if p<0.05

an IQR of 2.4–8.1%. The fever-phase group showed a median of 1.8% with an IQR of 0.5–2.85%. The critical-phase group showed a median of 3.6% with an IQR of 1.8–5.0%, while the median for the recovery-phase group was 7.05% with an IQR of 4.08–311.85%. The

convalescent-phase group showed a median of 7.3 % with an IQR of 3.95–9.3%, and the healthy-control group showed a median of 4.1% with an IQR of 2.2–6.6%.

The result of the statistical test using the Friedman test on IRF (%) in various

**Fig. Boxplot of IRF in Clinical Phases of Dengue Infection**

\*\*Note : analysis using a post hoc Mann Whitney test shows significance with p<0.05

**Table 2 Red Blood Cell Quantity and Immature Red Cell Fraction in Various Phases of Dengue Infection**

| Variable                                                  | Clinical Phase of Dengue Infection |                          |                          |                              | Healthy Control<br>n=27 | p* value            |
|-----------------------------------------------------------|------------------------------------|--------------------------|--------------------------|------------------------------|-------------------------|---------------------|
|                                                           | Fever Phase<br>(n=13)              | Critical Phase<br>(n=77) | Recovery Phase<br>(n=74) | Convalescent Phase<br>(n=53) |                         |                     |
| Red Blood Cells<br>(million/mm <sup>3</sup> )<br>mean±SD  | 4.88±0.64                          | 5.03±0.67                | 4.78±0.55                | 4.88±0.53                    | 5.02±0.53               | 0.103 <sup>a</sup>  |
| IRF (%)<br>median<br>IQR                                  | 1.80<br>0.50–2.85                  | 3.6<br>1.8–5.0           | 7.05<br>4.08–11.85       | 7.30<br>3.95–9.30            | 4.1<br>2.20–6.60        | <0.001 <sup>b</sup> |
| IRF Absolute<br>(x1000/mm <sup>3</sup> )<br>median<br>IQR | 93<br>25–141                       | 165<br>89–249            | 331<br>201–532           | 351<br>187–447               | 192<br>119–321          | <0.001 <sup>b</sup> |

\*Note : analysis using a: ANOVA test, b: Friedman test, c: Chi Square test, significant if p<0.05

clinical phases of dengue infection showed a significant difference with a p value of < 0.001. The statistical analysis was then followed by a *post hoc* Mann Whitney test to examine the difference between each group, and the difference is considered to be significant if p<0.05 (Fig).

The IRF (%) was lowest in the fever phase, then increased in the critical phase, reaching its highest in the recovery phase, and decreased in the convalescent phase. The IRF (%) in the fever phase was significantly different when compared to all the other phases and the healthy control group, with all the p value <0.05. The IRF (%) in the critical phase was significantly different when compared to the other phases in dengue infection (p<0.05), but not when compared to the healthy control group (p=0.218). The IRF (%) in the recovery phase was significantly different when compared to the fever phase, critical phase, and the healthy control group, but not when compared to the convalescent phase (p=0.629). The IRF (%) in the convalescent phase was significantly different when compared to the fever phase, critical phase, and the healthy control group (p<0.05). When compared to IRF (%), RBC counts were not significantly different among the phases of clinical dengue and also healthy control group.

## Discussion

There were a total of 244 research subjects, with the median age 24 years and IQR

(interquartile range) of 14–67 year. The number of male and female subjects was similar. The age and sex of the subjects in this research were consistent with the report from *Pusat Data dan Surveilans Kementerian Kesehatan Republik Indonesia* in 2010 stating that cases of dengue infection in Indonesia occurred predominantly in the age group of more than 15 years old with the distribution of the cases nearly the same in both sexes.<sup>1</sup>

The hemoglobin levels in this research were not significantly different among the groups. A study by La Russa concluded that the bone marrow suppression in dengue infection happened rapidly, that even though the bone marrow suppression affected all hematopoietic series, the substantial erythrocyte reserve can compensate for the decrease in erythrocyte production.<sup>3</sup>

The result of the statistical test on hematocrit levels in every group showed a significant difference between the groups, with the highest hematocrit level in the critical-phase group. The increase in hematocrit levels corresponds to the hemoconcentration and plasma leakage that happens during the critical phase. Malavige and Malasit suggested that the increase in capillary permeability was caused by the involvement of mediators such as TNF- $\alpha$ , IL-2, IL-8, and VEGF that would disrupt endothelial cells permeability *in vitro*. The intensity of the immune response and the titer of plasma viremia are the strongest independent factors for plasma leakage.<sup>12-13</sup>

The leucocyte counts among the groups were significantly different. The results

showed that the lowest leucocyte count was found in the fever-phase group, then increased gradually according to the disease phase. A study by Simmons, Na-Nakorn and LaRussa stated that leucopenia and neutropenia were found in the early phase of the disease, and a bone marrow biopsy in the early phase of the disease (less than five days of fever) also showed more hypocellularity.<sup>3,14</sup>

The statistical test on the variable of thrombocyte level showed significant difference between the groups. The thrombocyte level started decreasing in the fever phase, with the lowest level found in the critical phase and started increasing again in the recovery and convalescent phases. The result was consistent with the pathogenesis of dengue viral infection where the suppression of bone marrow happens in the early fever phase and reaches its lowest point in the critical phase.<sup>3</sup>

The IRF in the fever phase was the lowest compared with other phases of dengue infection. The IRF started rising in the critical phase and kept rising in the recovery and convalescent phases.

The IRF in the fever-phase group was significantly different compared with the other groups. The low IRF reflected the bone marrow suppression occurring in the fever phase of dengue infection. This result was consistent with the bone marrow biopsy research done by Simmons *et al* showing that the onset of bone marrow suppression could happen not more than 12 hours after the infection. A study by Noisakran also showed that the dengue virus can reach the bone marrow compartment in a short period of time, confirming that bone marrow suppression occurs in the fever phase.<sup>3,15</sup>

The analytical result of IRF in the critical phase showed a significant difference compared with other groups, except the healthy-control group. This showed that the recovery of bone marrow suppression has started in the critical phase. A study by Noisakran showed that the number of dengue virus RNA reached a peak in day 1 to day 3 after the infection and then the number decreased, so the bone marrow suppression by dengue virus RNA lessened in the critical phase (day 4 of sickness). Noisakran concluded that bone marrow recovery ended in day 10 (afebrile period) even though the blood cell destruction (especially thrombocyte) in the periphery reached its peak (owing to antibody and complement clearance).<sup>3,15</sup> A research by Clark KB also stated that a bone marrow

examination in day 4 to day 8 (critical phase) of sickness showed erythroid hyperplasia with maturation disruption.<sup>14</sup>

The IRF in the recovery-phase group was not significantly different compared to the convalescent-phase group. The IRF in the recovery-phase group and convalescent-phase group were higher than the critical-phase group and the healthy-control group. This result was similar with a bone marrow examination by Clark KB in day 10–14 of sickness that showed erythropoiesis hyperplasia without maturation disruption.<sup>14</sup>

The analytical result of IRF in the critical-phase group and healthy-control group using the Mann-Whitney test did not show any significant difference. The pathogenesis of dengue infection states that thrombocyte and leucocyte reach the lowest level in the critical phase, but the same thing did not happen to the erythrocyte in this research, represented by the IRF. This result was similar to research by Clark and Noisakran which showed that bone marrow suppression started its recovery in the critical phase, characterized by erythropoiesis hyperplasia with maturation disruption.<sup>3,14-15</sup> The increase in erythropoiesis is usually followed by an increase in erythropoietin. The life span of reticulocytes in the circulation also increases for up to three days or even more, due to the release of 'stress or shift' reticulocytes from the bone marrow and the acceleration of erythroid differentiation. 'Stress or shift' reticulocytes from the bone marrow have a large amount of RNA in their cells. The population of existing reticulocytes can not only be counted, the RNA content according to its maturity level needs to be assessed as well, and this has a significant clinical application in evaluating the erythropoiesis activity.<sup>16</sup>

The comparison between IRF in the critical-phase group and healthy-control group did not show significant difference, which shows that bone marrow of dengue patients in the critical phase is not different from the bone marrow of healthy people in general. This result confirmed that thrombocytopenia occurring in critical phase of dengue infection is caused by cell destruction in the periphery, due to the antibody and complement clearance.<sup>3</sup>

The previous retrospective study in 26 parvovirus B-19 infection in 119 sickle cell disease patients found that IRF is highly specific to detect severe aplasia. Increasing IRF predicts reticulocyte recovery in reticulocytopenia patients and could have utility in clinical decision making such as whether to transfuse packed red blood cells.<sup>17</sup>

In conclusion, this study was the first study describing IRF in multiple phases of dengue disease. There was an erythropoiesis difference between the phases of dengue infection. Bone marrow suppression for

erythropoiesis reached the lowest point in the fever phase and started recovering in the critical phase. Erythropoiesis in the critical phase was not different from the healthy control.

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