# The role of platelet antibody and bone marrow in adult dengue hemorrhagic fever with thrombocytopenia

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## Abstrak

   Kasus demam berdarah dengue (DBD) dewasa terus meningkat pesat pada saat ini. Di lain pihak penelitian yang dilaporkan terutama terjadi pada anak-anak. Selain itu, patofisiologi dan penatalaksanaan DBD pun masih kontroversial dan sering diperdebatkan. Telah dilakukan pemeriksaan aspirasi sumsum tulang, antibodi trombosit dan D-Dimer pada 29 pasien DBD dewasa (≥ 16 tahun). Diagnosis DBD ditegakkan berdasarkan kriteria klinis WHO, pemeriksaan imunokromatografi cepat dan diperkuat dengan pemeriksaan test IH, untuk memperjelas patogenesis trombositopenia dan patofisiologi DBD dewasa. Selain itu, dilakukan pemberian steroid dengan dosis imunosupresif selama 5 hari berturut-turut pada pasien tertentu secara double blind. Hasil penelitian menunjukkan bahwa pada demam/sakit < 5 hari, proses di perifer lebih berpengaruh untuk menyebabkan trombositopenia daripada penekanan sumsum tulang, dengan antibodi trombosit sebagai faktor yang paling berpengaruh (P=0.0112). Sedangkan pada demam/sakit > 5 hari tidak didapatkan variabel yang berpengaruh secara statistik. Untuk steroid, bila diberikan pada demam/sakit < 5 hari ternyata dapat mengurangi lama trombositopenia (P < 0.05), sedangkan pada demam/sakit > 5 hari ternyata hanya untuk mencegah penurunan tajam dari jumlah trombosit (P < 0.01). Kesimpulan: Trombositopenia pada orang dewasa terutama disebabkan oleh karena proses perifer ketimbang penekanan megakariosit sumsum tulang. Pada demam/sakit < 5 hari, steroid memperpendek lama trombositopenia dan mencegah penurunan tajam dari jumlah trombosit. Akan tetapi, pada demam/sakit > 5 hari, steroid hanya mencegah penurunan tajam dari jumlah trombosit.

## Abstract

   Adult  dengue haemorrhagic fever (DHF) cases are increasing rapidly, while most of the studies reported children cases. Controversies in DHF pathophysiology and treatment are still a matter of debate. In this study, bone marrow puncture, platelet antibody and D-Dimer examination were carried out in 29 adult DHF patient (more than 16 years). The diagnosis of DHF was confirmed clinically using the WHO criteria, rapid immunochromatographic examination supported by hemagglutination inhibition (HI) test to clarify the pathogenesis of thrombocytopenia and the pathophysiology of DHF in adult patient. Besides that, steroid was given in immunosuppressive dose during 5 days to selected patients (on double blind basis). The cross sectional study, cohort study and double blind trial were the methods used in this study. The results showed that periferal processes apparently were more affected than bone marrow suppression in causing thrombocytopenia, in the less than 5 day fever group, with platelet antibody as a leading factor (P = 0.0112). While, in the more than 5 day fever group, there are no significant variable. If steroid was given, in the less than 5 day fever group, apparently the length of thrombocytopenia could be decreased (P < 0.05), while the thrombocyte level could be kept from lowering too fast, in the more than 5 day fever group (P < 0.1). Conclusion: Most of thrombocytopenia was caused by periferal process than bone marrow suppression. In the less than five day fever group, the steroid could decrease the length of thrombocytopenia and keep the thrombocyte level from lowering too fast. However, in the more than 5 day fever group it only kept the thrombocyte level from lowering too fast.

Keywords: reticulo endothelial system, steroid, immunologic reaction

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Dengue Hemorrhagic Fever (DHF) is still an endemic illness in Indonesia. Today, DHF is endemic in 26 provinces (except Bengkulu).[1] In one of the surveys done by the Health Department of the Republic of Indonesia at nine cities, it was showed that Aedes aegypti was present in one third of the houses or public places investigated.[2] The latest data from the Health Department of the Republic of Indonesia, Aedes aegypti was found in more than 10% of the houses and buildings in 27 provinces.[1] Although national DHF

mortality rate is suppressed until 2.5%, mortality rate in hospitals caused by DHF at unexpected incidence in Indonesia is still high (5.7%).[1] In the other hand, the morbidity rate of adult DHF patients apparently was increased from 4.3% in 1968 to 40.56% in 1996.[1]

Thrombocytopenia is a central issue in the pathogenesis of DHF and bone marrow plays a role to cause that. It is supposed that suppression of bone marrow, especially the megakariocytes can cause thrombocytopenia in DHF patients with less than 5 day fever, while in patients with more than 5 day fever, thrombocytopenia is caused by peripheral process.[3,4] The spread of virus or viral antibody complex to bone marrow, was proved by Rothwell et al.[5] Bone marrow assesment data in adult DHF patient (more than 16 years) is still lacking. Most of bone marrow studies were carried out in children (less than 16 years).[6-10] Nelson and Bierman carried out bone marrow examinations in adult patients with hemorrhagic fever (not just dengue), but they did not explain whether the results were similar to DHF or not.[11] Clinical symptoms in children DHF patients are more dangerous than in adult patients. In children, thrombocytopenia was found in 82% and shock in 14% of patients.[12] However, in adult, thrombocytopenia was found only in 53.9% and shock in 1.4% of patients.[13] In 1995, the mortality rate for DHF at Cipto Mangunkusumo Hospital showed the same feature, i.e 3.6% in children and 0.8% in adult. Furthermore, there is no statistical analysis to determine the decrease of megakariocytes in bone marrow as a main cause of thrombocytopenia. Other causes of thrombocyte destruction in the periphery are disseminated intravascular coagulation (DIC), reticuloendothelial system (RES) activity, viral acitivity and immunologic reaction to destroy the immune complex that was adhered to the thrombocyte. Buthep and friends explained that endothelium destruction was a main cause of thrombocytopenia and leucopenia.[14] Funahara said that there were three leading peripheral factors to cause thrombocytopenia i.e. virus that destroyed the thrombocyte directly, endothelium destruction/consumptive coagulopathy and antigen antibody complex that destroyed the thrombocyte.[15] Radioisotope technology proved that RES activity was also a leading factor to cause thrombocytopenia.[16]

Platelet antibody can be positive in a DHF patient and played a role in causing thrombocytopenia. This is due to the increase of stimulation in autoreactive lymphocytes which is always present in human causing autoimmune reaction to thrombocyte. In the normal situation, body immunoregulator system always suc-

ceeds to prevent autoreactive lymphocytes in causing autoimmune reaction (normal immunology homeostasis mechanism). Isarangkura and Tuchinda proved that thrombocyte transfusion to DHF patients with severe thrombocytopenia showed no significant result.[17]

Until now, it is supposed that the thrombocytes are not the target cell of dengue virus. The target cells of dengue virus are monocytes, macrophages and Kupffer cells.[18] Therefore, the adhesion of immune complex (virus antibody complex) to endothelial wall, thrombocytes, and kidney cells, even to brain and pancreas cells was more likely to be body over reaction.[19,20] Halstead, the leader of the secondary heterologous infection theory, changed his opinion to genetic theory to explain the severe clinical symptom that could happen in primary infection, occured in Europe, United States and Australia.[21,22] Myomin and Futrakul gave steroid to dengue shock syndrome (DSS) patients and got a good result, but Sumarmo in his two studies (1973 and 1978) did not find a significant result.[23,24]

In this study, we tried to give steroid to the patients. This is according to an assumption that in an adult DHF patient the bone marrow was less affected compared to the peripheral process (immunologic reaction). Besides that, the possibility of positive platelet antibody, and hypersensitivity reaction stage III as DHF basic pathophisiology still need a consideration.

## METHODS

### Study design

1. Cross sectional study. This design is useful to find out the role of bone marrow and the influence of the other variables in causing low thrombocyte count at the time of admission.
2. Cohort study. This is useful to evaluate thrombocytopenia until it reach the normal level and the influence of platelet antibody and steroid on thrombocyte count.
3. Double blind test. This is useful to know the effect of steroid on thrombocyte count.

### *Inclusion criteria to this study*

Patients over 16 years which were clinically proved as DHF, according to the WHO criteria,[25] except the increase in hematocrit value. In the WHO criteria, the increase in hematocrit value is based on the increase above the normal value according to age, sex and population. In Indonesia, there is no data about that.

The inclusion criteria are: Fever (or history of acute fever) less than 8 days (2-7 days); thrombocyte level <100,000/mm3; haemorrhagic tendencies (minimally Rumple Leede +) and other criteria to prevent bias thrombocytopenia i.e. normal spleen (physical finding), chest x-ray and urine analysis.

The patients were divided into two groups, the group with fever less than 5 days and the group with fever more than 5 days.

### Exclusion criteria for this study

All patients with negative IgG or IgM on rapid immunochromatography, or patients whose second hemagglutination inhibition (HI) test level did not increase four times compared to the first HI test or whose HI test was not more than 1/2560 (presumtive positive) are ecluded from this study.

### Double blind test grouping

We divided all sample into two groups, i.e. the group of patients receiving steroid and fluid treatment (10 patients) and patients receiving fluid treatment and placebo. Then we analyzed the results in the group receiving steroid compared to the group receiving placebo, in the less than 5 day and more than 5 day fever group.

### Exclusion criteria for steroid administration

The patient with gastrointestinal bleeding and whose thrombocyte level is lowered to 20.000/mm3 in a routine examination was excluded from receiving steroid.

### Investigation procedure

The patients were subjected to bone marrow puncture and blood collection for haematologic, serologic and D-Dimer investigation and viral isolation. Some patients were subjected to steroid administration scheme.

The increase of thrombocyte level in all patients was observed day by day. The patient was sent home if the thrombocyte level was more than 100.000/mm3 in two subsequent days; but before that, the blood sample should be taken for the second HI test. For a patient with positive D-Dimer, the blood sample will be taken everyday until we found a negative D-Dimer.

## Laboratory procedures

### Rapid immunochromatography

The principle is to know if there's any antibody to dengue virus type 1, 2, 3, 4, in the patient. We used the patient's serum (from 3 ml of venous blood) and applied it on nitrocellulose paper containing dengue virus antigen. Positive rapid immunochromatography test showed the change of color to the color of control IgG and/or IgM box. The instrument was adjusted to the WHO criteria based on positive HI test for IgG and IgM. Therefore, a secondary infection showed changes of color in the IgG and IgM boxes.

### Assessment of bone marrow cell

The bone marrow puncture (BMP) was carried out as fast as possible, in 24 hours or more, and before the administration of steroid. The bone marrow megakariocytes were calculated and its morphology were analyzed. The number of megakariocyte is considered normal when we found 12-50 megakaryocytes/50 low magnification field. This is the normal value used in the Division of Hematology Department of Internal Medicine, Cipto Mangunkusumo Hospital. According to other investigators the bottom limits are 0 or 11 and the upper limits are 27 or 38.[26-30] Besides that, the differential counts of 200 bone marrow cells were done.

### Haematologic investigation

We took 2 1/2 ml of venous blood and added 2 mg of EDTA. The sample was sent for complete blood count i.e. Hb, Ht, and leucocyte and thrombocyte count, immediately to the Clinical Pathology Laboratory Cipto Mangunkusumo/Persahabatan Hospital (for patients who came at night) or to the Hematology Laboratory Cipto Mangunkusumo Hospital for patient who came in the morning. The instrument used were Coulter Max M (Clinical Pathology Laboratory) and Micro Coba S (Hematology Laboratory).

### Serologic investigation

1. Inhibition hemaglutination test. This test was based on the fact that dengue virus had the ability to aglutinate red blood cells, and the specific antibody in the patient's serum would inhibit the aglutination process. We took 3 ml of venous blood, put it in vacutainer tube and sent the samples immediately (within 4 hours) to the Health Research and Development Insitution of NAMRU-2 Laboratory. The HI test were conducted when the patient came

and leaved the hospital. The positive result was shown by a four time increase in HI level.

2. Platelet antibody examination was carried out by immunofluorescent method.

### D-dimer investigation

We took 4.5 ml of venous blood, added 1 ml of 3.8% sodium citrate and the samples were centrifuged (using RC sorval 3 type centrifuge) at 4°C and 2000 x g for 10 minutes. The plasm was put into chromotimer Behring. This investigation was carried out at the Hematology Laboratory Cipto Mangunkusumo Hospital.

### Viral isolation

Viral isolation of dengue virus was carried out at NAMRU-2 or the Microbiology Laboratory University of Indonesia. The procedure was: patient's serum inoculation to the intrathorax of female/ male *Aedes aegypti*, incubation between 10-13 days at 30°C with relative humidity, and detection of dengue antigen. The detection of dengue antigen was done at the end of incubation time, i.e. the mosquito was killed and the head of the mosquito were reacted against anti-dengue antibody which was conjugated to a fluorescent dye (isotiocyanat) and screened using fluorescence microscopy. The presence of the virus grown in the mosquito could be seen as green-yellow fluorecent particle.

To isolate the dengue virus, we took 3 ml of venous blood and put it into a vacutainer tube and sent it to the lab in less than 4 hours. Viral isolation was stopped if most of the results from the 30 patients were negative.

### Steroid administration scheme

The steroid used was Medixon® (metil prednisolon) in immunosuppresive dose, 1.2-1.5 mg/kg body weight/day, during 5 days.

### Statistical analysis methods

Statistical analysis (chi-square, thrombocytopenia relative risk and multiple regression analysis) was performed using SPSS program for windows (6.0).

## RESULTS

There were 33 cases from May through July 1997, which were collected in Cipto Mangunkusumo and Persahabatan General Hospital, and 29 of them were included in the study. The entire result of this study is shown in Table 1, 2 and 3 and figure 1.

Table 1. The result bone marrow cell assessment

| Assessment | Fever 2-4 days (11 patients) | % | Fever 5-7 days (18 patients) | % |
|---|---|---|---|---|
| Bone marrow puncture | 11 | 100 | 17 | 94 |
| Hypocellular | 7 | 63.6 | 2 | 11.8 |
| Normocellular | 4 | 36.4 | 10 | 58.9 |
| Hypercelllular | 0 | - | 5 | 29.3 |
| Decrease of megakariocyte | 6 | 54.6 | 3 | 17.6 |
| Normal megakariocyte | 5 | 45.4 | 9 | 52.9 |
| Increase of megakariocyte | 0 | - | 5 | 29.5 |
| Young form megakariocyte | 8 | 72.1 | 4 | 25.5 |
| Decrease of leukocyte | 7 | 63.6 | 6 | 35.9 |
| Normal leukocyte count | 4 | 36.4 | 11 | 64.1 |

Table 2. The result of hematologic investigation

| Assessment | Fever 2-4 days (11 patients) | % | Fever 5-7 days (18 patients) | % |
|---|---|---|---|---|
| Leukocytosis | 0 | 0 | 0 | 0 |
| Leukopenia | 9 | 81.8 | 11 | 68.2 |
| Average of first leukocyte count (Le 0) | 3500/µl | - | 4300/µl | - |
| Average of first thrombocyte count (TR 0) | 59.000/mm3 | - | 43.000/mm3 | - |

Table 3. The result of physical finding, serologic and D-dimer investigation, viral isolation, and steroid administration

| Assessment | Fever 2-4 days (11 patients) | % | Fever 5-7 days (18 patients) | % |
|---|---|---|---|---|
| Fever (+) | 11 | 100 | 2 | 23 |
| Real bleeding (+) | 3 | 27 | 2 | 11 |
| Platelet antibody (+) | 7 | 63.6 | 11 | 61.6 |
| D-dimer : | | | | |
| DIC (+) | 6 | 54.5 | 5 | 27.7 |
| DIC NOT Com (+) | 1 | 9 | 1 | 5 |
| Virus isolation (+) | 0 | 0 | 0 | 0 |
| Steroid (+) | 2 | 18.2 | 8 | 47 |

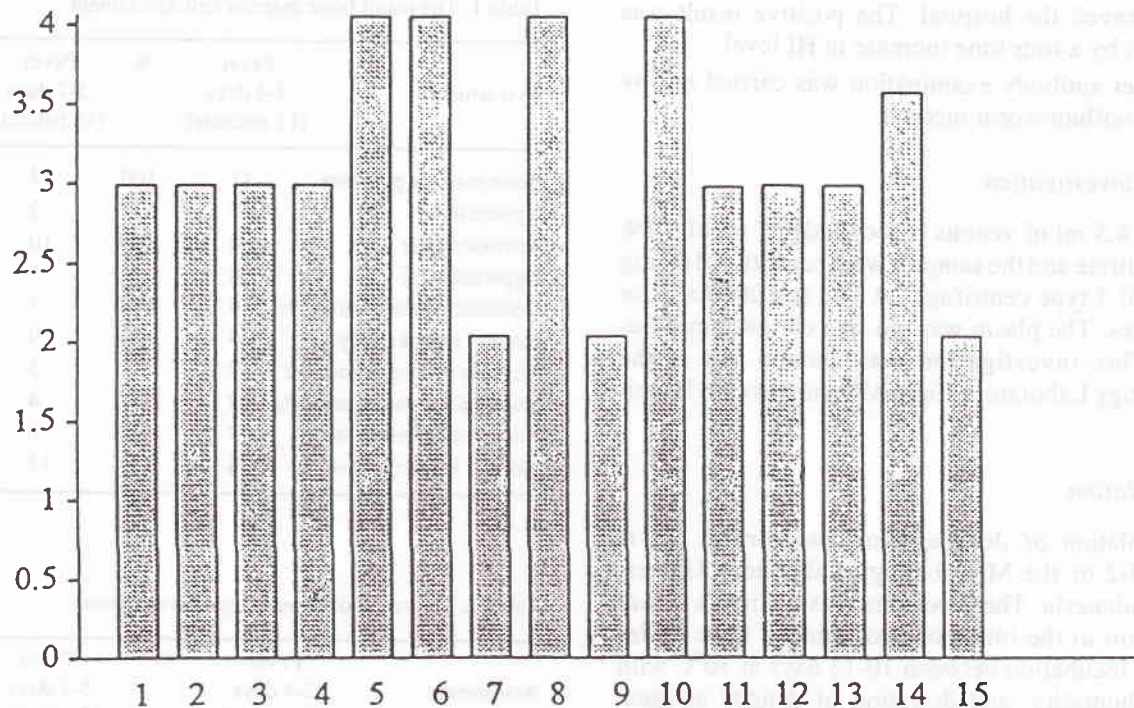DIC = disseminated intravascular coagulation, DIC not com = decompensated DIC

*Figure 1. The length of thrombocytopenia in the 2-4 day fever group and 5-7 day fever group*

LT= the length of thrombocytopenia, Abtr= platelet antibody,  str= steroid administration

Figure information:

| LT of 27 patients | 2-4 day fever group | 5-7 day fever group |
|---|---|---|
| 1. Average | 6. Average | 11. Average |
| 2. Abtr (+), Str (+) | 7. Abtr (+), Str (+) | 12. Abtr (+), Str (+) |
| 3. Abtr (-), Str (-) | 8. Abtr (-), Str (-) | 13. Abtr (-), Str (-) |
| 4. Abtr (-), Str (+) | 9. Abtr (-), Str (+) | 14. Abtr (-), Str (+) |
| 5. Abtr (+). Str (-) | 10. Abtr (+), Str (-) | 15. Abtr (+), Str (-) |

The thrombocyte count decreased excessively in four patients with positive platelet antibody, after they achieved 100.000/mm3. Those four patients did not get steroid. They represented 40% of the 10 patients with positive platelet antibody and without steroid administration.

There were four patients with thrombocytopenia that lacked the variable causing thrombocytopenia (negative platelet antibody, normal megakariocyte in bone marrow assessment, no fever or viremia, no bleeding, and negative DIC).

## Statistical analysis

### Multiple regression analysis

Multiple regression analysis was used to analyze the variables influencing the first thrombocyte count (TR 0). In the less than 5 day fever group, all variables (platelet antibody, decrease in bone marrow mega-kariocytes, decompensated DIC, and real bleeding) showed significant influence ($P<0.05$ ) on TR 0, with platelet antibody as a leading factor ($P= 0.0112$). The order of the influencing variable are platelet antibody, decompesated DIC, real bleeding and decrease in bone marrow megakariocytes. However, multiple regression analysis to analyze the variables influencing TR 0 in the more than 5 day fever group showed that no variable had significant influence. Furthermore one way multiple regression analysis of the entire patients (29 cases) showed that apparently only platelet antibody has significant influence on TR 0 ($P= 0.09$).

### Thrombocytopenia relative risk analysis

The relative risk of thrombocytopenia in patients with platelet antibody (+) was increased 1.5, 1.06 and 1.75

times (confidence limits 95%) in the less than 5-day fever group, more than 5 day fever group and in entire patients respectively, compared to the patients with negative platelet antibody.

*Chi-square analysis*

Chi-square analysis showed a significant difference in the duration of thrombocytopenia between patients with and without steroid administration ($P < 0.05$), in the less than 5 day fever group, while in the more than 5 day fever group, there was no significant difference between patients with and without steroid administration. One way chi-square analysis showed that platelet antibody might be the cause of the fast decrease in thrombocyte level ($P = 0.0568$). Furthermore, one way chi-square analysis showed that steroid administration to patients with positive platelet antibody in the two groups might keep the thrombocyte level from lowering too fast ($P = 0.07364$).

## DISCUSSION

In the less than 5 day fever group, positive platelet antibody was found in 62.5% of the cases and the quite high influence of decompensated DIC to cause early thrombocytopenia, lead to the conclusion that positive platelet antibody and decompensated DIC could be regarded as a leading factor in causing thrombocytopenia. Furthermore, by seeing the strength of positive platelet antibody influence to cause early thrombocytopenia in the less than 5 day fever group ($P = 0.0012$) and the higher relative risk in developing thrombocytopenia in this group (1.5 times), we concluded that the bone marrow suppression was not the main cause of thrombocytopenia.

The role of steroid administration in the less than 5 day fever group to decrease the duration of thrombocytopenia was evaluated, even it was only given in two patients. Actually, there were four patients who received the steroid in this group, but two patients were excluded from the study, because the result of rapid immunochromatographic and HI test were negative, even, obviously the clinical symptoms were DHF. The negative serology in those two patients needed further consideration because steroid administration was given too early (in the first and second day of the illness). This condition could inhibit the formation of IgG and IgM, resulting in negative serologi. Steroid administration decreased the length of thrombocytopenia significantly ($P = 0.01850$).

In the more than 5 day fever group, there were no variable that influence the first thrombocyte count (the number of thrombocyte at the time before BMP). However, RES activity needed to be considered as the cause of early thrombocytopenia in this group. This was based on the four patients who developed thrombocytopenia without influencing variables causing thrombocytopenia. The average thrombocyte count in those four patients was 33,000. The occurrence of increased RES activity to destroy the thrombocytes that were invested by immune complex was already proved by radioisotope. On the other hand, the low relative risk of the patients with positive platelet antibody compared to negative platelet antibody to develop thrombocytopenia, was due to the immunoregulator that succeeded to neutralize the autoreactive lymphocytes, after 5 days. The decreased of autoreactive lymphocytes was caused by the decrease of the stimulation caused by the immune complex, because a lot of immune complex were destroyed. Furthermore, the condition of positive platelet antibody became negative, as a consequence of the decrease in autoimmune reaction.

In the more than 5 day fever group, steroid had no effect on the duration of thrombocytopenia. Steroid administration only slowed down the lowering of thrombocyte level. This was due to the inhibition effect of steroid on the immunoregulator system. Steroid inhibited autoreactive lymphocytes i.e. T helper cell (Th) to give wrong impulse to B lymphocytes. Time was needed to take over the regulation of Th lymphocyte (autoreactive lymphocyte) by the steroid, so that duration of thrombocytopenia was not decreased. In the other hand, steroid was statistically proved to keep the thrombocyte level from lowering too fast which was caused by the presence of platelet antibody.

## CONCLUSION

1. Periferal process was more affected than bone marrow suppression to cause thrombocytopenia in the less than 5 day fever group, with platelet antibody as a leading factor.

2. In the more than 5 day fever group, the influence of RES activity as a cause of thrombocytopenia needs to be considered.

3. Steroid administration in the less than 5 day fever group could shorten the duration of thrombocytopenia, but in the more than 5 day fever group, the effect was only to keep thrombocyte level from lowering too fast.

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