The Kinetics of CD8+ T Lymphocytes in Dengue Patients in Yogyakarta

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ABSTRACT

Introduction: Dengue fever can be graded into dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The CD8+ T lymphocytes mediate antiviral activity by producing cytokines and directly destroyed the dengue virus infected cells. This study focuses in observing the kinetics of CD8+ T lymphocytes absolute and relative count in dengue patients.
Objectives: To observe the kinetics of CD8+ T lymphocytes absolute and relative count in dengue patients.
Methods: The research design used is a descriptive study. This research measures and observes the kinetics CD8+ T lymphocytes absolute and relative count from day 2 to day 7. The CD8+ T lymphocytes count was determined using flowcytometry. Data was analyzed using ANOVA and independent t test with $p<0.05$ considered as significant.
Results: The CD8+ T lymphocytes absolute count is low during the beginning of disease course and it gradually increases from day 2 to day 7. The CD8+ T lymphocytes relative count decreases from day 2 to day 3, and start to increase back from day 3 to day 7. There is no difference between the level of CD8+ T lymphocytes absolute count and relative count between DF and DHF patients.
Conclusion: There is an increase in CD8+ T lymphocytes absolute count and relative count in dengue patients. There is no difference between DF and DHF patients in CD8+ T lymphocytes absolute and relative count.

Keywords: dengue fever; dengue hemorrhagic fever; CD8+ T lymphocytes; absolute count; relative count.

INTISARI

Pendahuluan: Demam Dengue dapat digolongkan menjadi Demam Dengue (*Dengue Fever*/DF), Demam Berdarah Dengue (*Dengue Hemorragic Fever*/DHF) dan Sindrom Syok Dengue (*Dengue Shock Syndrome*/DSS). Limfosit T CD8+ T memediasi aktivitas antiviral dengan menghasilkan sitokin-sitokin dan menghancurkan sel yang terinfeksi virus dengue secara langsung. Penelitian ini mempelajari perubahan-perubahan yang terjadi pada jumlah limfosit T CD8+ pada pasien dengue.
Tujuan: Mempelajari kinetika limfosit T CD8+ yaitu hitung absolut dan relatif pada pasien-pasien Dengue.
Methods: Desain penelitian ini adalah penelitian deskriptif. Pada penelitian ini, diukur dan diamati kinetika limfosit T CD8+ yaitu jumlah absolut dan relatif pada hari ke 2 sampai hari ke 7. Hitung limfosit T CD8+ ditentukan dengan *flowsitometry*. Data dianalisis dengan Uji ANOVA dan Uji t dengan batas signifikan $p<0,05$.

Hasil: Hitung absolut limfosit T CD8+ rendah pada permulaan perjalanan penyakit tetapi secara bertahap meningkat dari hari ke 2 sampai hari ke 7. Hitung relatif limfosit T CD8+ T menurun dari hari ke 2 sampai hari ke 3 dan meningkat kembali dari hari ke 3 sampai hari ke 7. Tidak terdapat perbedaan antara hitung absolut dan relatif limfosit T CD8+ antara pasien-pasien DF dan DHF.
Simpulan: Terdapat peningkatan hitung absolut dan relatif limfosit T CD8+. Tidak terdapat perbedaan antara hitung absolut dan relatif limfosit T CD8+ antara pasien-pasien DF dan DHF.

Kata Kunci: demam dengue; demam berdarah dengue; Limfosit T CD8+; hitung absolut; hitung relatif

INTRODUCTION

Dengue viruses are mosquito-borne, transmitted through infected female *Aedes* mosquito, causing dengue. Dengue viruses, belonging to family of *Flaviviridae*, are positive-strand RNA viruses. There are four subtypes of dengue virus, which are DENV-1, 2, 3 and 4, each composing of similar antigen. As such, life-long immunity acquired after being infected by one of the dengue virus does not serves immunity as well for the remaining dengue viruses[1].

Dengue virus is covered by a lipid bilayer with 2 proteins designated as envelope (E) and membrane-associated (M) proteins. The E protein is the protein involved in immune system, whereby it functions by binding to cellular receptors, then fuses with cell membrane. Neutralizing antibodies produced from immune system will recognize the epitopes in the E protein, thus induce the immunity against dengue viruses. The exact mechanism is still under investigation[2].

The dengue viruses are transmitted by female *Aedes* mosquito. The incubation period of dengue viruses is 4.5-7 days, rarely exceeding 10 days[3]. Infected individuals will only show sign and symptoms of dengue infection after this period. The sign and symptoms that appeared last for 3-10 days. Infected individuals are contagious and prone to spread the virus to the mosquitoes when the blood contains high load of viruses, period that starts slightly before the sign and symptoms appear, which lasts for 5 days[4]. These mosquitoes that transmit diseases, known as vectors, carry the dengue virus throughout the life after biting an infecting[5]. The virus in the mosquito will be further incubated for 8-12 days in its body before it is virulent to individuals who are bitten[4].

Currently, dengue is estimated to have infected 2.5 billion people, circulating in endemic area and periodically caused widespread epidemics. Dengue is endemic in nearly 100 countries, consisting of Asia, Pacific, America, Africa and Caribbean. Estimation has been made that there are new incidence of dengue infection, 50-100 millions occurred yearly, ended with 22,000 deaths due to dengue infection[6].

Situated in Asia, Indonesia has high prevalence of dengue cases, with the number of cases increasing yearly. In 1998, Indonesia recorded 72,133 cases of dengue fever (DF) and dengue hemorrhagic fever (DHF). During that year, dengue occurs in 56 countries which in total more than 1.2 million cases reported to WHO[6].

According to report from Regional Office of South East Asia, Thailand has the highest number of cases until 2003. From 2004 onwards, Indonesia took over as the highest and the trend of cases increases rapidly, with Indonesia alone achieve 57% of cases in 2006[7].

From 1 January to 30 April 2004, according to WHO, 58,301 cases of dengue infection was reported to Indonesian Ministry of Health with 658 deaths due to dengue infection. The current predominant affecting virus is DENV-3 (37%), as compared to the remaining three dengue viruses[8]. According to the 1993-1994 outbreak reported in Papua, DENV-3 is the predominant serotype. This is due to fact that this dengue virus serotype is recognized in many cases of DHF during the outbreak compare to other strainsof viruses such as DENV-1 and DENV-2[9].

Among 30 affected provinces such as Jakarta, Bali and East Nusa Tenggara, 17 of provinces reported to have outbreaks of dengue with unusually high numbers of cases[8]. In year 2007, WHO received reports that there were 150,000 of dengue cases, where 25,000 of these cases are from Jakarta and West Java[6].

The exact mechanism of pathogenesis of dengue virus is still not clearly defined. Several hypotheses have been proposed and the most recent hypothesis is the serotype cross-reactive T lymphocytes. In this theory, the CD8+ T lymphocytes secrete cytokines that alter the endothelial cells and cause plasma leakage, resulting in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The cytokines also play an important role in lysing dengue viruses-infected monocyte[10].

The CD8+ T lymphocytes play a major role againts viruses. The body produced CD8+ T lymphocytes in order to fight against viruses. Produced by the body in response to viral antigen, CD8+ T lymphocytes aims to kill the infected cells and prevent further spread of viral infection. Asides from CD8+ T lymphocytes, CD4+ T lymphocytes also play a critical role in immune system of the body in dengue infection. CD4+ T lymphocytes are needed to generate CD8+ T lymphocytes continuously and antibody. It also secretes lymphokines, a substance that can attract and activate macrophage and natural killer (NK) cells. With these mechanisms, body immune system can fight to protect against dengue virus[11]. Previous research reported that the level of soluble CD4 increases during viremia, with soluble CD8 level increases soon after the period. This indicates that CD4+ T cells are activated during viremia and CD8+ T cells are activated after CD4+ T cells are activated first[12].

The CD8+ T lymphocytes also contribute in protecting body during dengue virus infection. CD8+ T lymphocytes mediate the antiviral activity by producing cytokines such as interferon-gamma and tumor necrosis factor – alpha, besides direct killing the infected cells[13]. The lack of researches regarding the role of CD8+ T lymphocytes in dengue virus stress the need to further explore the relationship between CD8+ T lymphocytes and dengue viruses. This study focuses in observing the kinetics of CD8+ T lymphocytes in dengue patients and investigating the correlation of the kinetics with dengue severity.

MATERIALS AND METHODS

This study is a descriptive study, conducted in Dr. Sardjito Hospital from April to May 2009. The study is done to observe the kinetics of CD8+ T lymphocyte in dengue patients. The subjects used in this study are

patients aged above 14 years old with the diagnosis of dengue fever using the WHO criteria (1997), and are admitted in Dr. Sardjito Hospital.

The inclusion criterias are patients admitted in Dr. Sardjito Hospital, > 14 years old, diagnosed to have dengue infection with NS-1 positive (+ve), had fever in the first and second day and had signed informed consent. The exclusion criterias are patients who also suffered from other diseases, other than dengue infection, or dropped out due to loss of follow up and contracted with fever not due to dengue infection.

Using the vacutainer tubes with K_3EDTA, 3 mL of venous blood is withdrawn from the anterior cubiti vein. The blood withdrawn is used as sample. The specimen used is stored in room temperature and examined within 24 hours.

First, 50 µL of whole blood specimen obtained from patient is inserted into a tube. Next, 10 µL of Tritest reagent CD8 FITC-HLA-DR and PE/CD45 are added into the tube. Then, the specimen is mixed well with vortex mixer and incubated in a dark room with room temperature for 15 minutes. After that, 450 µL of lysing solution is added into the tube. Lastly, the specimen is mixed well and incubated in a dark room at room temperature for 15 minutes again. The CD8+ T lymphocytes absolute count of the sample is measured using fluorescence activated cell sorter (FACS).

Dengue is classified based on WHO criteria (1997)[14]. The day patient admitted to hospital for observation until the day being discharge from hospital is recorded. CD8+ T lymphocytes absolute count is the number of CD8+ T lymphocytes present in blood. CD8+ T lymphocytes relative count is the percentage of CD8+ T lymphocytes out of the total population of cells

With the data obtained, mean of absolute count and relative count of CD8+ T lymphocytes number for each day is calculated and presented in graph. Data was analyzed using ANOVA and independent t test. ANOVA is conducted to determine the significance of the mean differences between each day. Independent t-test is performed to analyze the significance of the mean differences between DF and DHF for each day. If the result $p<0.05$, the mean differences is significant. If the result $p>0.05$, the mean differences is not significant.

RESULTS AND DISCUSSION

There were a total of 32 participants in this study. From these 32 participants, only 28 are included in this study since the data of 4 participants are incomplete. Among these 28 participants, there was equal distribution of participants with 14 of them each suffered dengue fever (DF) and dengue hemorrhagic fever (DHF) respectively. For the DF participants, 9 of them were male while 5 of them were female. On the other hand, DHF participants consisted of 8 males and 6 females.

The research focused on the condition of participants from day 2 until day 7, measuring the absolute count and relative count of CD8+ T lymphocytes. During data analysis, there were some data that were not present. Hence, the number of data obtained for each day slightly differed. At the same time, as some of the data were incomplete and had insufficient numbers of data, day 1 and day 8 were excluded from the data analysis. This was because statistical analysis would not be valid if data of day 1 and day 8 were included. In this study, the data distribution were observed using boxplot. If the data distribution were not normally distributed, a transformation is conducted to have a normal curve.

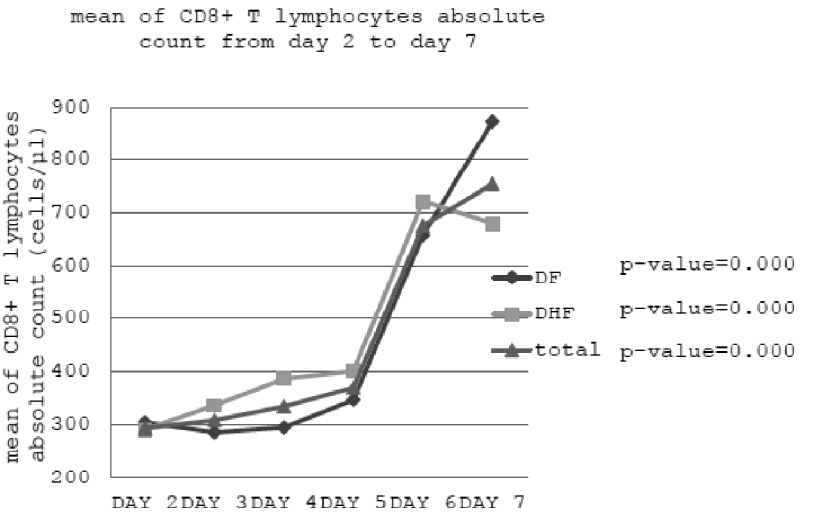

Figure 1. Graph showing day to day mean of CD8+ T lymphocytes absolute count from day 2 to day 7

A. CD8+ T lymphocytes absolute count

Figure 1 shows mean of absolute count of CD8+ T lymphocytes which increased from day 2 until day 7 generally in both dengue fever (DF) and dengue hemorrhagic fever (DHF) patients. The normal range of absolute count of CD8+ T lymphocytes used in this study is according to MultiSET Software from United States of America which is 190 cells/µL to 1140 cells/µL[15].

In DF patients, the absolute count is low from day 2, then it gradually increases until day 5 (mean 346.62cells/µl ± 288.80). After that, there was a sharp increase from day 5 to day 6, and continued to rise in day 7 (mean 872.37cells/µl ± 317.07). As determined by ANOVA, there was a statistically significant difference between mean number of CD8+ T lymphocytes absolute count (p-value =0.000). The CD8+ T lymphocytes absolute count significantly increases in day 6 (658.25 cells/µl ± 382.651, p=0.021) and in day 7 (872.37 cells/µl ± 317.071, p=0.000) compared to day 2.

On the other hand, in DHF patients, the absolute count gradually increased from day 2 until day 5. Then, there was an abrupt rise in day 6 (722.36cells/µl ± 408.78), but then it declined slighty in day 7 (608.16cells/µl ± 140.88). There is a statistically significant difference between mean of CD8+ T lymphocytes absolute count (p = 0.000). The decline in day 7 was not significant (p = 1.000).

Sarasombath et al. (1988) found out that the absolute CD8+ cells in the study conducted among 61 children aged 8 months to 12 years decreased in the febrile stage.[16] In fact, the lowest amount of CD8+ cells absolute count was recorded to be on the first day of defeverscence. After that, the CD8+ cells rise quickly and reached beyond the normal value on the second day of defeverscence, follow by gradual decrease of absolute count back to normal range.

Study by Kurane et al. (1991) that measured the soluble CD8 (sCD8) suggested that there was no elevation of the level of sCD8 in the sera of

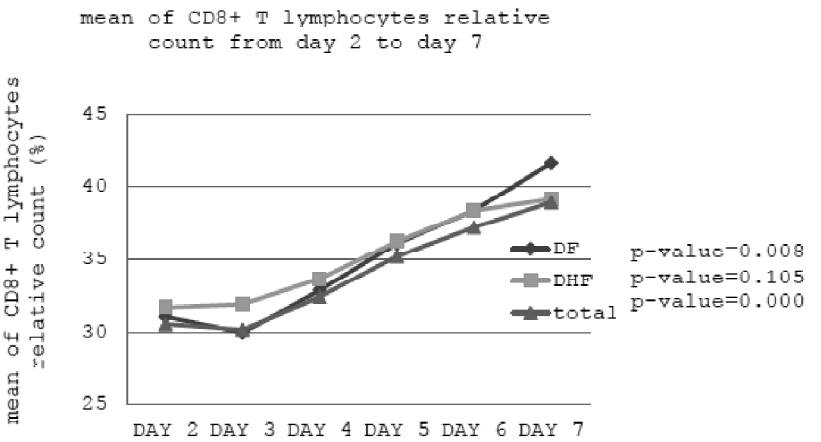

Figure 2. Graph showing day to day mean of CD8+ T lymphocytes relative count from day 2 to day 7

DF patents during days 1-20[17]. Their study also found out that the levels of sCD8 were higher in DHF patients on days 3-20 after onset of fever in comparison to DF patients. DHF patients show highest level of sCD8 in days 5-6.

Fadilah et al. in Malaysia recruited 109 patients with fever who were suscpected to have DHF in 1999[18]. They found out that the absolute count of CD8 cells reduces in DHF patients. CD8+ cells are low, differentiating DHF from DF patients in the fever phase of dengue viral infection.

In year 2006, Azeredo et al. has a study which investigate 60 dengue patients in Brazil[19]. This study shows that there is a decrease in absolute CD8+ T cell counts in dengue patients in comparison to controls. The level of CD8 was reduced during acute phase.

B. CD8+ T lymphocytes relative count

Figure 3 shows the mean of CD8+ T lympho-cytes relative count increased from day 2 until day 7 generally in both dengue fever and dengue hemorrhagic fever patients. The normal range of CD8+ T lymphocytes relative count used in this study is according to MultiSET Software from United States of America, which is 13% - 41%[15].

In dengue fever, the relative count dropped slightly from day 2 to day 3, then it steadily increased from day 3 (29.95% ± 8.59) until day 7 (41.64% ± 7.44). As determined by ANOVA, there was a statistically significant difference between mean of CD8+ T lymphocytes relative count (p=0.008). The mean relative count of CD8+ T lymphocytes was statistically significantly higher in day 7 (41.63% ± 7.24, p=0.038) compared to day 2.

On the other hand, dengue hemorrhagic fever patients' relative count gradually increases for each day from day 2 (mean 31.69% ± 7.74) until day 7 (39.18% ± 8.32). ANOVA analysis shows there was no statistically significant difference between groups of mean of CD8+ T lymphocytes relative count (p=0.105) in dengue hemorrhagic fever.

In 2006, Mongkolsapaya et al. conducted a research among children aged 8 to 10 years

in Thailand, which aims to investigate T lymphocytes responses in dengue viral infection[20]. Their research results shows that the frequency of CD8+ T lymphocytes rise to low levels in acute phase, then peak in 2 weeks during samples followed up.

According to Friberg et al. (2011) the level of CD8+ T cells were high during acute phase and also in the days shortly after the fever of patients subsides, the period known as early convalescence[21]. They also observed changes in the CD8+ T lymphocytes in DF and DHF for both primary and secondary infection of dengue.

The research conducted by Mladinich et al. shows that percentage of CD8 activation increases and peak in day 14[22]. Mladnich et al. also proposed that higher viral loads causes higher level of T lymphocytes expansion, hence causing increase in disease severity. Dung et al. conducted a research in year 2010, with the result showing the percentage of CD8+ T lymphocytes were most prominent after defervescence[23].

C. Comparison of CD8+ T lymphocytes between DF and DHF

Independent t-test was performed to compare the CD8+ T lymphocytes absolute and relative count between DF and DHF. The analysis did not show any significant difference in any days between DF and DHF in both absolute count and relative count ($p > 0.05$). These findings indicate that the CD8+ T lymphocytes absolute and relative count were not significantly related with the severity of dengue virus infection.

Previously reported by Kurane et al. (1991), the activation of T lymphocytes is greater in DHF than in DF, with significant correlation between severity of disease and increase level of sCD8[17]. This research suggests that high levels of T cell activation may be related to the pathogenesis of DHF.

This is supported by research conducted in Thailand by Green et al., whereby the level of sCD8 were significantly higher in children with DHF compared to those with DF[24]. The difference in the findings may be due to the fact that both the researches were conducted among children in Thailand. On the other hand, this study is performed in Yogyakarta among adults.

Research by Mongkolsapaya et al. showed that the magnitude T cell responses were related to disease severity[20]. Their research in year 2006 clearly showed significantly higher level of T cell responses in DHF patients in comparison with patients who had DF and normal group patients. The results of this research have led to a theory of T cell response involving in dengue pathogenesis. This may be due to the contribution of activated T lymphocytes to severe plasma leakage. Entrance of virus into monocytes and macrophages causes viral peptides to be presented on cell surface. These antigen-presenting cells interacted with memory T lymphocytes and induce proliferation and production of proinflammatory cytokines, including TNF-alpha and IFN-gamma. These cytokines directly affect vascular endothelial cells and causes plasma leakage. Hence, it is predicted that DHF patients would have higher level of T lymphocytes activation[25].

Simmons et al. in 2005 found out that the magnitude of T cell responses were not significantly related with disease severity[26]. Analyzing Vietnamese adults, their research result showed that there was no association between T cell response and dengue severity.

However, there are several limitations in this study. This study only involved a limited number

of participants. The duration of the study is also limited to a week, whereby no follow up is conducted.

CONCLUSION

The CD8+ T lymphocytes absolute count is low during the beginning of disease course and it gradually increases from day 2 to day 7. The CD8+ T lymphocytes relative count decreases from day 2 to day 3, and start to increase back from day 3 to day 7. There is no difference between the level of CD8+ T lymphocytes absolute count and relative count between DF and DHF.

SUGGESTION

The result of the study would be more accurate if there are more participants involved in the study, with wider range in age. The outcome would be more favorable as well if the duration of the study, which is from day 2 until day 7 can be lengthened to obtain more accurate result.

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