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Current Issue

Jul-Dec 2022 - Volume 5 - Issue 2

Editor-in-Chief: Prof. Muhammad Miftahussurur, MD., Ph.D
Online ISSN: 2620-8636
Frequency: Two issues per year

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Original Article

Total Leukocyte Count in *Rattus norvegicus* after Duffy Binding-Like 2β-*Plasmodium falciparum* Erythrocyte Membrane Protein 1 Recombinant Protein Injection: The way to a Peptide-based Malaria Vaccine Development

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Submitted: 05-Sep-2022
Revised: 14-Sep-2022
Accepted: 22-Sep-2022
Published: 09-Dec-2022

ABSTRACT

Introduction: Severe malaria caused by *Plasmodium falciparum* is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). It has a DBL2β domain that specifically binds to the intercellular adhesion molecule-1 (ICAM-1) receptor that lies in endothelial cells of many vital organs and is involved in malaria pathogenesis. Antibody against the DBL2β-PfEMP1 protein correlates with a reduced risk of severe malaria, making it a potential malaria vaccine candidate. This study aimed to examine total leukocytes after serial DBL2β-PfEMP1 recombinant protein injection to determine its immunogenicity. Settings and Design: This was an experimental study using pre-post control groups design. **Methods:** Samples were male rats aged 2-3 months with a weight of 150-350 g. Rats were injected 3x with 100 µg, 150 µg, and 200 µg of the purified DBL2β-PfEMP1 recombinant protein in the three-weeks interval. Blood samples were collected on days 0, 8, 29, and 50, and total leukocytes were counted using the improved Neubauer counting chamber and observed under a microscope. The data were analyzed using the Friedman test, Kruskal Wallis test, and Mann-Whitney test. **Results:** The lowest leukocyte level was at the pre-injection, and the highest level was after the third injection. There was a significant increase in leukocytes ($P < 0.05$) in all treatment groups but no increase in the control group. A dose of 100 µg of DBL2β-PfEMP1 recombinant protein showed the best response in inducing the increase of total leukocytes. **Conclusion:** The DBL2β-PfEMP1 recombinant protein could induce the higher leukocyte level in each injection, especially after the third injection.

Keywords: *Duffy binding-like domain, leukocyte, malaria vaccine candidate, protein*

Introduction

Malaria, a disease caused by the *Plasmodium* parasite and transmitted by the female *Anopheles* mosquito, is a major health problem. World Health Organization reported approximately 229 million malaria cases in 2019, with an estimated death number of 409,000 cases. As much as 63% of all malaria cases in Indonesia were caused by *Plasmodium falciparum,* the rest were caused by *Plasmodium vivax*, and only a small portion was caused by *Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*.[1,2] Severe malaria due to *P. falciparum* is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) that is expressed on the surface of infected red blood cells (iRBCs).[2] The adhesion between iRBCs and vascular endothelial cell receptors is known as the cytoadherence mechanism, which may occur in the microcirculation of vital organs.[3]

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Quick Response Code:

Website: https://journals.lww.com/bhsj

DOI: 10.4103/bhsj.bhsj_30_22

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How to cite this article: Putri DA, Sulistyaningsih E, Kusuma IF, Dewi R. Total leukocyte count in *Rattus norvegicus* after Duffy binding-like 2β-*Plasmodium falciparum* erythrocyte membrane protein 1 recombinant protein injection: The way to a peptide-based malaria vaccine development. Biomol Health Sci J 2022;5:71-6.

This mechanism will cause damage to various related organs. Furthermore, there is the adhesion of iRBC to normal erythrocytes, called a rosetting mechanism.[4] In addition to inhibiting circulation or blood flow in infected vital organs, the cytoadherence and rosetting mechanisms will prevent iRBC from being destroyed in the spleen.[5]

The PfEMP1 has a Duffy binding-like (DBL) domain for binding to its receptors. The DBL2β domain, a type of DBL domain, can bind intercellular adhesion molecule-1 (ICAM-1) and is associated with severe malaria.[6,7] Study has shown that the antibody against the DBL2β recombinant protein may block the binding between DBL-2β and the ICAM-1 receptor and reduce the risk of severe malaria.[8]

Malaria vaccine can be an essential approach for combating malaria, but the development of malaria vaccine is challenging due to the parasite's complexity. A peptide-based vaccine could be an alternative. Several proteins were studied as malaria vaccine targets, including PfEMP-1. The essential role of the DBL-2β-PfEMP-1 in the pathogenesis of severe malaria makes it a candidate for a peptide-based malaria vaccine.[9]

Vaccine-candidate protein should have immunogenic properties, meaning has the capacity to induce an immune response. One indicator of protein immunogenicity is the increase of leukocyte number after exposure to protein. A leukocyte is a protective unit that plays a vital role in the immune system to provoke an immune response. It depicts an immune response that becomes a defense against foreign substances.[10] Leukocytes may represent either the innate or adaptive immune systems that have a role in cell-mediated activities, humoral activities, and memory cell activity that prevents reinfection.[11] Leukocytes can do phagocytosis, and antigen recognition, destroy pathogens through cytotoxic-induced apoptosis and start a complement cascade to lyse the pathogen.[10] The increase in leukocytes also increases antibodies which cause the body's immunity to be stronger.[12] In this study, the total leukocytes were counted after serial injection of three different doses of the DBL2β-PfEMP1 recombinant protein to determine its immunogenicity.

Methods

Ethical clearance

The study received ethical approval from The Ethical Committee of the Faculty of Medicine, the University of Jember, with reference number No. 1560/H25.1.11/KE/2022.

Study design and animal sample

It was a true experimental design with a randomized pre-and posttest control group design. As many as 16 male Wistar rats (*Rattus norvegicus*), age 8–10 weeks, with a bodyweight of approximately 150–300 g, were provided for the study as calculated by resource equation methods. Rats were acclimatized at laboratory conditions with 30°C–35°C with a 12-h light cycle and standard feeding for 2 weeks.

The Duffy binding-like-2β-*Plasmodium falciparum* erythrocyte membrane protein 1 recombinant protein production and purification

The production of DBL-2β-PfEMP-1 recombinant protein was performed using the procedure conducted by Rachmania *et al*. (2020).[9] The DBL-2β-PfEMP1 recombinant protein was expressed in *Escherichia coli* BL21 (DE3). It started with culturing the DBL2β-PfEMP1 clone cells in *Luria-Bertani* media containing kanamycin at 37°C with a shaker incubator at 150 rpm until it reached an optical density with a wavelength of 600 nm 0.6–0.8. The expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was harvested, and the DBL-2β-PfEMP-1 recombinant protein was extracted by lysozyme addition and sonication.

The recombinant protein was subsequently purified based on affinity chromatography using Ni-NTA agarose, using an elution buffer containing 60- and 100-mM imidazole, respectively. The purified recombinant protein was further analyzed for its concentration using the Bradford protein assay and electrophorized with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 12.5% in 50 V for 45 min for upper gel and 80 V for 120 min for lower gel. The gel was stained using Coomassie brilliant blue.

Experimental design

After 2 weeks of acclimatization, 16 male rats were randomly divided into control groups and three treatment groups. A control group was injected with 0.9% NaCl, and the three treatment groups were injected subcutaneously with 100, 150, and 200 μg of purified DBL-2β-PfEMP-1 recombinant protein, respectively. Complete Freund's adjuvant and incomplete Freund's adjuvant were added to the protein using a ratio of 1:1 for primary and secondary injection, respectively. The injection was delivered subcutaneously on days 0, 21, and 42. The sera were collected using sterile capillaire on an orbital vein on days 0, 8, 29, and 50 for total leukocyte counts.

Total leukocyte counts

The total leukocyte counts were conducted using the improved Neubauer counting chamber with a 1:20 dilution of Turk solution and observed under a × 10 and × 40 microscope. The calculation was performed using the formula:

Leukocytes count per mm^3 = (number of counted cells/volume of squares) × dilution factor.

Data analysis

The data analysis was performed with Statistical Package for The Social Sciences (SPSS) version 24.0 IBM, Armonk, New York, United States using the Friedman test, Kruskal–Wallis test, and Mann–Whitney test due to its normality and homogeneity tests with the significance value of 5% and $P < 0.05$ was considered statistically significant. The Friedman test was conducted to examine the significant difference between the preinjection and postinjection. The difference of leukocyte count among groups was determined with Kruskal–Wallis test then continued by Mann–Whitney pairwise comparison to analyze the significance between groups.

Results

The Duffy binding-like-2β-*Plasmodium falciparum* erythrocyte membrane protein 1 recombinant protein production and purification

The target of DBL-2β-PfEMP-1 recombinant protein was ~72 kDa, as in the previous study.[9] Visualization using SDS-PAGE confirmed it, and the highest concentration was obtained at the second round elution of 60-mM imidazole (E2.2) [Figure 1].

Leukocyte count

Figure 2 shows the average leukocyte counts in the control and treatment groups on pre-and serial postinjection of different doses of purified DBL-2β-PfEMP-1 recombinant protein. There was an increase in the average leukocyte counts in the treatment group injected with different doses of purified DBL-2β-PfEMP-1 protein. The leukocyte counts increased progressively with injection frequency, with the highest increase of leukocyte counts reached after the third recombinant protein injection. Furthermore, a dose of 100 μg of purified DBL-2β-PfEMP-1 recombinant protein showed the highest increase in leukocyte count.

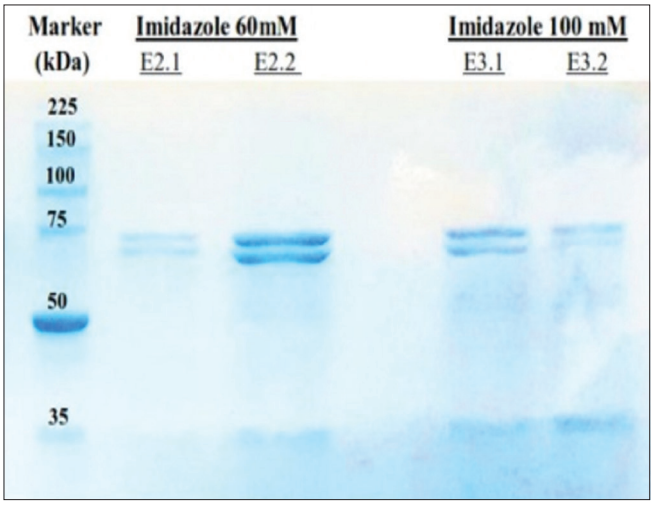

Figure 1: The DBL-2β-PfEMP-1 protein is visualized as a ~72 kDa band. DBL-2β: Duffy binding-like-2β, PfEMP-1: *Plasmodium falciparum* erythrocyte membrane protein 1

Data analysis

The data analysis showed a normal distribution ($P > 0.05$) in the Shapiro–Wilk test but was not homogeneous in the Levene test ($P < 0.05$). The Friedman test analysis on the difference in leukocyte counts on pre- and postinjection of purified DBL-2β-PfEMP-1 recombinant protein showed a significant difference between groups ($P = 0.000$). The test also reported that the rank of average leukocyte counts from the lowest to the highest was preinjection (day 0), postfirst injection (day 8), postsecond injection (day 29), and postthird injection (day 50). Furthermore, an analysis of the difference between the control group and treatment groups was performed using the Kruskal–Wallis test showed no significant difference between groups at preinjection and postfirst injection. However, the Mann–Whitney test showed a significant difference in postsecond and third injections [Figure 3]. The Mann–Whitney test of the group's difference is shown in Figure 3. After the first protein injection, only the dose of 150 μg recombinant protein differed from the control group. Meanwhile, after the second and third protein injection, all doses showed a significant difference from the control group. Furthermore, there was no difference between treatment groups in all injection periods.

Discussion

An increase of total leukocytes in blood circulation is a sign of the body's immune response to fight the pathogen. The study showed a significant increase in total leukocytes after injection of the purified

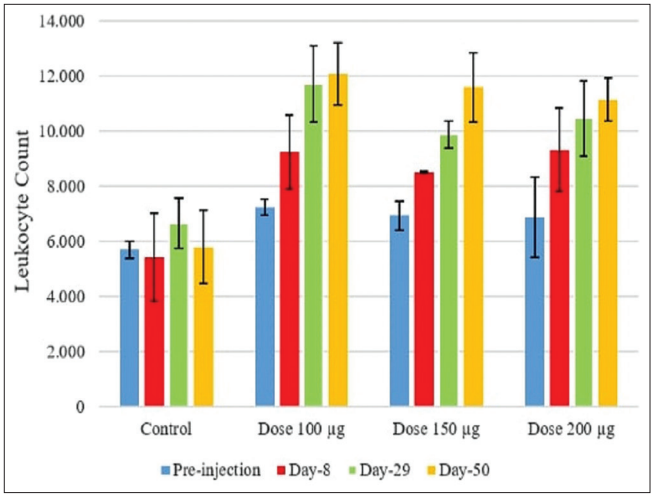

Figure 2: The comparison of average leukocyte count in control and three treatment groups (doses of 100, 150, and 200 μg purified DBL-2β-PfEMP-1 recombinant protein) on preinjection (Day 0), Day 8, 29, and 50. DBL-2β: PfEMP-1. DBL-2β: Duffy binding-like-2β, PfEMP-1: *Plasmodium falciparum* erythrocyte membrane protein 1

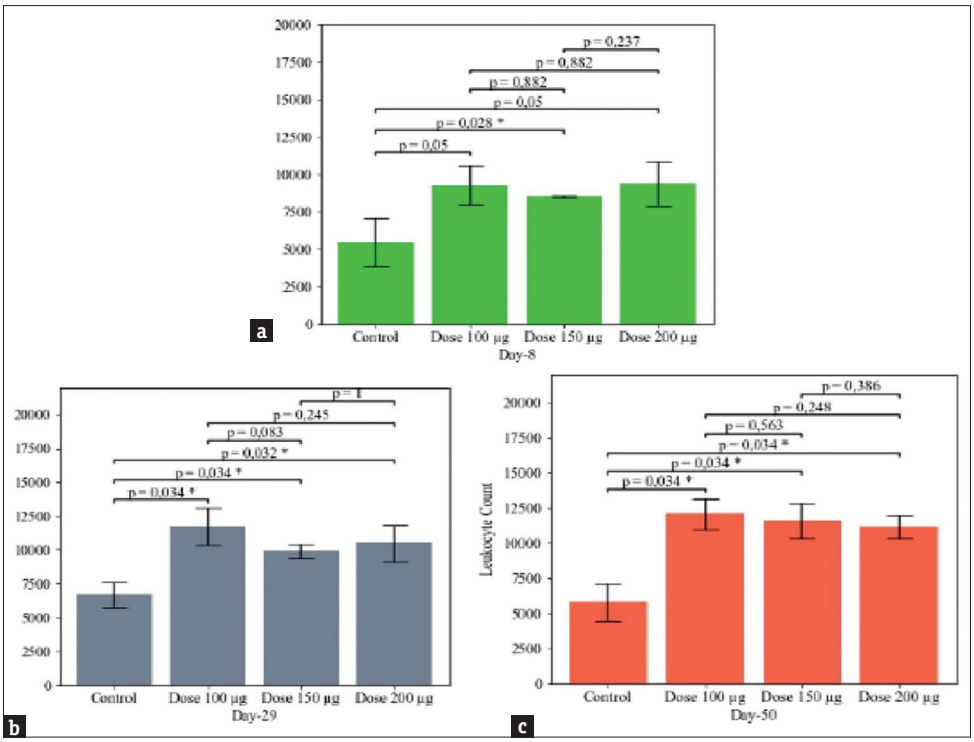

Figure 3: (a) Day 8 (b) Day 29 (c) Day 50. The leukocyte count was measured in triplicate. The data were analyzed using the Mann–Whitney test with the significance level (*) of $P < 0.05$. DBL-2β: Duffy binding-like-2β, PfEMP-1: *Plasmodium falciparum* erythrocyte membrane protein 1

DBL-2β-PfEMP-1 recombinant protein (P = 0.000). The Friedman test demonstrated that the lowest average of total leukocytes was at preinjection. The total leukocytes increased with the frequency of purified DBL-2β-PfEMP-1 recombinant protein injection. Studies reported that repeated immunogenic protein injection strengthens the immune response. The DBL-2β-PfEMP-1 recombinant protein is a protein candidate for a subunit malaria vaccine prepared by recombinant DNA technology.[10] The subunit vaccine requires repeated injections to stimulate the immune response. The subunit type of vaccine does not produce an immune response as robust as a vaccine containing a live-attenuated virus or pathogen.[13] Study on live-attenuated blood-stage malaria vaccine still challenging due to significant hurdles in completing initial testing in the human subject and also issues related to the safety, longevity of immune response, production's scalability, logistic storage, and distribution.[14] A new approach of malaria vaccine design based on protein could be an important solution to solve the problems. The complexity of the *Plasmodium* life cycle makes the malaria vaccine development targeting on the specific stage of *Plasmodium* which causes pathogenesis, such as preerythrocytic stage, erythrocytic or blood stage, and transmission-blocking vaccine. Those vaccines could induce an immune response and prevent the mortality of malaria.[15-17] The previous study using another subunit malarial vaccine candidate reported that repeated injections strengthen the immune response, which is indicated by high antibody titers after the third injection.[18] Commensurable to this study, the leukocytes increased after repeated injections.

The upsurge of leukocytes ensued in treatment groups but did not transpire in the control group. The normal total leukocyte of rats (*R. norvegicus*) is 5000–13000 cells/mm^3.[14] Before injection, the leukocyte count of rats in this study varied between 5000 and 8000 cells/mm^3, and it increased along with the frequency of protein injection up to 9600–13300 cells/mm^3 after the third injection as presented in the treatment groups. There was no significant difference in total leukocytes between groups (P = 0.283) on day 0 before protein injection. Meanwhile, there was a significantly different on the days 29 and 50. Furthermore, there was a significant difference in total leukocytes within all treatment groups [Table 1], indicating the remarkable increase in total leukocytes after the second and third protein injection. This is in accordance with previous studies that report a robust immune response and repeated injection. The injected DBL-2β-PfEMP-1 recombinant protein will be recognized as an antigen by antigen-presenting cells,[9] which further activate leukocytes, particularly B lymphocytes and T lymphocytes,[15,16] by clonal expansion and differentiation that reach the peak

Table 1: The average of leukocyte counts in control and treatment groups and the significance within group and between groups

Group	Leukocyte count Average±SD (sel/mm^3)				Significance within group (*P*)
	Day 0 (preinjection)	Day 8 (post 1st injection)	Day 29 (post 2nd injection)	Day 50 (post 3rd injection)	
Control group (0.9% NaCl)	5700±308	5433±1.608	6667±920	5800±1.327	0.532
100 µg recombinant protein	7238±288	9250±1.337	11,725±1.393	12,088±1.112	0.026*
150 µg recombinant protein	6938±532	8525±43	9900±489	11,600±1.243	0.007*
200 µg recombinant protein	6875±1.464	9338±1.506	10,475±1.365	11,150±785	0.038*
Significance between group (*P*)	0.283	0.072	0.015**	0.030**	

*There are significant differences ($P<0.05$) within group, **There are significant differences ($P<0.05$) between group. The leukocyte counts were measured in triplicate and the data were analyzed using the Kruskal-Wallis test. SD: Standard deviation

in 7 days postantigen exposure.[17,19] Following the activation phase, the cellular and humoral immunity work as an effector phase which aims to eliminate antigens. Cellular immunity is mediated by helper T lymphocytes and cytotoxic T lymphocytes and plays an essential role in aiding macrophages to eliminate antigens and allowing B cells to produce antibodies and helper T-cells also secrete cytokines that may increase the number of leukocytes.[17]

Previous studies on malaria vaccine candidates using merozoite surface protein-3 (MSP-3) in children showed that various doses led to disparities in inducing leukocyte counts.[20] The increase of leukocytes was observed at a dose of 15 µg, while the increased dose up to 30 µg decreased leukocyte count.[20] In this study, a significant increase of leukocytes after the first protein injection was observed at a dose of 150 µg only. However, all doses showed a significant increase in leukocytes after the second and third injections [Figure 3]. Meanwhile, a dose of 100 µg of DBL-2β-PfEMP-1 recombinant protein resulted in the highest total leukocyte after the second and third protein injections [Table 1]. The high total leukocyte indicated a high immune response. Therefore, a dose of 100 µg of DBL-2β-PfEMP1 recombinant protein is suggested as the best dose to induce an immune response.

A vaccine candidate should be capable of inducing immune response either in primary or booster injection or both. A previous study using circumsporozoite protein as a vaccine candidate showed a significant increase in leukocytes along with injection frequency.[18] In this study, the injection of purified DBL2β-PfEMP1 recombinant protein resulted in an increase of total leukocytes, which indicated the immunogenicity of the protein and could be a malaria vaccine candidate.[21-27]

Conclusion

This study indicated that the DBL-2β-PfEMP-1 recombinant protein induces the immune response demonstrated by the increase of leukocytes in rats and a dose of 100 µg of DBL-2β-PfEMP-1 recombinant protein was the best dose in inducing the increase of leukocytes. The ability of the DBL-2β-PfEMP-1 recombinant protein to induce a rat immune response suggested the potential of this protein as a candidate for a peptide-based malaria vaccine.

Acknowledgments

The author would like to thank the University of Jember for funding the study through Hibah Keris Malaria 2021 (Malaria Research Group Grant, 2021) No. 2662/UN25.3.1/LT/2021.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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