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DBL2β-PfEMP1 Recombinant Protein Dose Effect on Inducing Humoral and Cellular Immune Responses in Wistar Rats: A Study on Malaria Vaccine Development

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Abstract: The DBL2β-PfEMP1 is a promising malaria vaccine candidate because of its essential role in malaria pathogenesis. This study aimed to determine the effective dose of DBL2β-PfEMP1 recombinant protein in inducing humoral and cellular immune responses by measuring immunoglobulin M (IgM), immunoglobulin G (IgG), and a cluster of differentiation 4 (CD4+) concentration in Wistar rats. The DBL2β-PfEMP1 recombinant protein was produced in *Escherichia coli* BL21(DE3) and purified by affinity chromatography using Ni-NTA resin. Wistar rats (*Rattus norvegicus*) weighing 150–200 g were randomly divided into control and three treatment groups. A control group was subcutaneously injected with 0.9% NaCl, and treatment groups were injected with 100, 150, and 200 μg of purified DBL2β-PfEMP1 recombinant protein three times at 3-week intervals. Sera were collected two weeks after each injection for immune response measurement using enzyme-linked immunosorbent assay (ELISA). Data were statistically analyzed using mixed-method ANOVA followed by a post hoc Bonferroni test. IgM and CD4+ concentrations consistently increased in all treatment groups after consecutive injections. However, IgG showed a slightly different result. The highest levels of IgM, IgG, and CD4+ were observed at a dose of 200 μg after the third injection. Statistical analysis confirmed the difference between the control and treatment groups with p=0.000, 0.034, and 0.006 for IgM, IgG, and CD4+. These findings imply that in the dose range of the study, a dose of 200 μg DBL2β-PfEMP1 recombinant protein resulted in the highest response to induce humoral and cellular immunity and could be an effective dose for malaria vaccine.

Keywords: immune response, malaria, PfEMP1, vaccine.

DBL2β-电磁脉冲 1 重组蛋白对诱导维斯塔大鼠体液和细胞免疫反应的剂量效应：疟疾疫苗开发研究

摘要：DBL2β-电磁脉冲 1 是一种有前途的疟疾候选疫苗，因为它在疟疾发病机制中发挥着重要作用。本研究旨在通过测量维斯塔大鼠的免疫球蛋白中号(免疫球蛋白中号)、免疫球蛋白 G (免疫球蛋白 G)和分化簇 4 (光盘 4+)浓度来确定 DBL2β-电磁脉冲 1 重组蛋白诱导体

液和细胞免疫反应的有效剂量。DBL2β-电磁脉冲 1 重组蛋白在大肠杆菌 BL21(德 3)中产生，并使用镍-国家税务局树脂通过亲和层析纯化。体重 150-200 克的维斯塔大鼠（褐家鼠）被随机分为对照组和三个治疗组。对照组皮下注射 0.9%氯化钠，治疗组皮下注射 100、150 和 200 微克纯化的 DBL2β-电磁脉冲 1 重组蛋白，注射 3 次，间隔 3 周。每次注射两周后收集血清，使用酶联免疫吸附测定（酶联免疫吸附试验）测量免疫反应。使用混合方法方差分析和事后邦费罗尼检验对数据进行统计分析。连续注射后，所有治疗组的免疫球蛋白中号和光盘 4+浓度持续增加。然而，免疫球蛋白 G 显示的结果略有不同。第三次注射后，在 200 微克剂量时观察到免疫球蛋白中号、免疫球蛋白 G 和光盘 4+水平最高。统计分析证实了对照组和治疗组之间免疫球蛋白中号、免疫球蛋白 G 和光盘 4+的差异，p=0.000、0.034 和 0.006。这些发现表明，在研究的剂量范围内，200 微克 DBL2β-电磁脉冲 1 重组蛋白的剂量可产生最高的诱导体液和细胞免疫反应，可能是疟疾疫苗的有效剂量。

关键词：免疫反应、疟疾、电磁脉冲 1、疫苗。

1. Introduction

Malaria is an endemic infectious disease in tropical and subtropical countries, causing approximately 247 million clinical cases and 619,000 deaths worldwide [1]. *Plasmodium falciparum* causes the highest malaria mortality, with a percentage reaching 99% [2, 3]. Malaria vaccine development is pivotal to reducing malaria incidence and mortality. In 2015, the Malaria Vaccine Initiative (VMI) launched the first-generation malaria vaccine based on circumsporozoite protein (CSP), i.e., RTS,S/AS01. A Phase-3 trial (2009-2014) was conducted in Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and Tanzania on children aged 5–17 months, who had received three doses of RTS,S/AS01, and a booster showed only 26-36% efficacy against severe malaria [4, 5]. Therefore, malaria vaccine candidates using different protein targets still need to be developed.

An important target of vaccine development is the blood stage of *Plasmodium falciparum*, where vaccines aim to prevent merozoite invasion of erythrocytes and severe clinical symptoms. Antibodies against merozoites defeat parasite blood stage replication and disease by numerous mechanisms, including direct inhibition of erythrocyte invasion, interaction with complement to lyse merozoites and inhibit invasion, and interaction with Fcγ receptors to promote phagocytosis [4]. One of the blood stage proteins studied as vaccine candidates is *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1).

PfEMP1 is an antigenic protein expressed during the erythrocytic cycle on the surface of infected erythrocytes (IEs). The protein is encoded by a highly diverse *var* gene family [6]. The head structure of the PfEMP1 extracellular domain consists of several cysteine-rich interdomain regions (CIDR) and the Duffy binding-like (DBL) domains that contribute to severe malaria pathogenesis. DBL2β domain can bind to intercellular adhesion molecule-1 (ICAM-1) receptor, inducing cytoadherence to avoid IE destruction. However, cytoadherence could lead to the development of severe malaria complications such as cerebral malaria. The crucial importance of DBL2β-PfEMP1 for parasite survival makes this antigen a major immune target, and gaining PfEMP1-specific antibodies is a central component of naturally acquired protection [7–9]. Therefore, the DBL2β-PfEMP1 becomes a protein target for malaria vaccine development because a potential vaccine candidate should be able to generate an adequate immune response to counter the infection.

Falciparum malaria infection generates humoral and cellular immune responses that work synergistically to control the infection severity [10, 11]. PfEMP1-specific antibody inhibited merozoite invasion and cytoadherence [11]. An *in vivo* study of DBL2β-PfEMP1 recombinant protein showed the induction of specific immunoglobulin G (IgG) and a cluster of differentiation 4 (CD4+) production [12], while the DBLα-tag-specific CD4+ contributed to protecting children from subsequent malaria episodes [13]. Studies have indicated that immunoglobulin and CD4+ levels could be representative parameters for assessing the immune response to DBL2β-PfEMP1.

Immunoglobulin G plays a pivotal role in immunity against malaria infection [14–16]. The higher the IgG level, the lower the severity of malaria [16]. IgG binds to epitopes and prevents interaction between the antigen and receptor on the host cell. It inhibits merozoite invasion and opsonization to mediate

phagocytosis and activates the complement system, which results in phagocytosis [15].

In malaria infection, IgM is produced by plasma B cells and first expressed after exposure to a foreign antigen. Studies have also reported that specific IgM not only is rapidly induced following a primary *P. falciparum* infection in malaria-naive adults but also is prominent in most patients during malaria disease and *P. falciparum* infection. IgM is a long-lived response in the absence of reinfection. It can also inhibit merozoite invasion of erythrocytes and blood-stage replication by the fixation and activation of complement on the merozoite surface [17–19]. In addition, CD4+ is the main target and mediator of the immunoregulatory pathway established during malaria infection. CD4+ plays a vital role in priming phagocytic cells to capture and kill the parasites and in B cell activation to produce anti-malarial antibodies [11].

Our previous study demonstrated that the DBL2β-PfEMP1 recombinant protein induces humoral and cellular immune responses by measuring IgG and CD4+ levels using only a single protein dose [12]. A recent study was conducted using several doses of DBL2β-PfEMP1 recombinant protein to determine the effective dose as a malaria vaccine candidate by measuring IgM, IgG, and CD4+ levels.

2. Materials and Methods

2.1. Production and Purification of DBL2β-PfEMP1 Recombinant Protein

Transformed *E. coli* BL21(DE3) were cultured in LB medium supplemented with 50 μg/mL kanamycin by shaking at 190 rpm, room temperature (RT), to reach an optical density OD_{600} of 0.6–0.8 and induced with 0.5 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) by shaking at 190 rpm, RT, for 8 h. The cells were harvested, and pellet cells were solubilized with extraction buffer containing 500 mM NaCl, 50 mM NaH_2PO_4, and 50 mM imidazole (pH 8.0). Solubilized pellet cells were added with 1 mg/mL lysozyme, incubated at 4°C for 30 min, and subsequently sonicated. The soluble fraction was collected by centrifugation at 12,000 rpm at 4°C for 30 min [12].

Purification was carried out using affinity chromatography based on the Ni-NTA resin kit protocol. Briefly, 4 mL of the soluble fraction was added to a column containing 1 mL of Ni-NTA resin and washed with 2.5 mL of wash buffer and different concentrations of imidazole, i.e., 10 and 20 mM. Each concentration was washed twice. The DBL2β-PfEMP1 recombinant protein was eluted using 0.5 mL of an elution buffer and different concentrations of imidazole, i.e., 30, 60, and 100 mM (pH 8.0); each concentration was eluted twice [12].

2.2. The Bradford Protein Assay

The protein concentration was measured using the Bradford protein assay with bovine serum albumin (BSA) as a standard. The standard protein and Bradford reagent were prepared according to the manufacturer's protocol to generate the standard curve equation. As much as 5 μL of purified protein was added to 995 μL of Bradford reagent and subsequently incubated at room temperature for 10 min. The absorbance was measured using a spectrophotometer at 595 nm [12].

2.3. Visualization of DBL2β-PfEMP1 Recombinant Protein

The purified proteins were visualized in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Bio-Rad Laboratories, USA). Running buffer containing 0.025 M Tris base, 0.1% SDS, and 0.192 M glycine and sample buffer containing 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% glycerol, 0.5% bromophenol blue, and 5% β-mercaptoethanol were prepared. The proteins were denatured at 95°C for 5 min using a dry heat block and run in 4.5% upper gel at 50 V for 1 h and 12.5% lower gel at 80 V for 3 h. The gel was stained using a staining solution containing 0.1% (w/v) CBB, 50% (v/v) methanol, 10% (v/v) acetic acid, and 40% (v/v) aquadest and subsequently destained using a destaining solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, and 50% (v/v) aquadest [20].

2.4. Immunization of DBL2β-PfEMP1 Recombinant Protein in Wistar Rats

Fifteen male Wistar rats (*Rattus norvegicus*) weighing 150–200 g, aged 2-3 months, were randomly divided into a control group and three treatment groups. The rats were acclimatized for 2 weeks under laboratory conditions at 30-35°C, 12-h light cycle, and standard feeding. Rats in the control group were subcutaneously injected with 0.9% NaCl, and the three treatment groups were injected with 100, 150, and 200 μg purified DBL2β-PfEMP1 recombinant protein, respectively. Injections were performed 3 times with an interval of 3 weeks (Days 0, 21, and 42). Complete Freund's adjuvant was emulsified with recombinant protein in the first injection and incomplete Freund's adjuvant in the subsequent two injections. Serum was collected from all groups from the orbital vein using sterile capillaries 2 weeks after injection (Days 14, 35, and 56) [21].

2.5. Measurement of IgM, IgG, and CD4+ Concentrations by Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA plate was coated with antigen and blocking buffer at a serum-coating buffer ratio of 1:1 (100 μL). The plate was wrapped in aluminum foil and incubated at 4°C for 16 h. After incubation, the blocking buffer was removed, and the plate was washed with 100 μL washing buffer (PBS-Tweens) for

3 min with 3 repetitions. Furthermore, 100 µL of antibody conjugate was put into the plate and incubated at room temperature for 2 h before being discarded and washed with 200 µL of PBS Tween for 3 repetitions. A total of 100 µL of a substrate containing peroxide and chromogen was put into the plate in the dark, incubated at room temperature for 15 min, and subsequently added with 50 µL of stop solution (H_2SO_4) and incubated for 10 min. The results were read using a microplate reader at 450 nm 3 times every 5 min [22–24].

2.6. Statistical Analysis

IgM, IgG, and CD4+ levels were analyzed using IBM SPSS Statistics 26. Normality and homogeneity were analyzed using the Shapiro-Wilk and Levene tests ($p>0.05$). The differences in IgM, IgG, and CD4+ levels among the groups were analyzed using mixed-method ANOVA followed by a post hoc Bonferroni test with a 95% confidence interval.

2.7. Ethical Approval

This study was approved by the Ethics Committee of the Faculty of Dentistry and Faculty of Medicine, University of Jember, East Java, Indonesia, with reference number 1536/UN25.8/KEPK/DL/2021.

3. Results

3.1. Recombinant Protein Visualization

The DBL2β-PfEMP1 recombinant protein was visualized as a ~72-kDa thick band through SDS-PAGE, with the most prominent band shown in 60 mM imidazole elusion, as presented in Fig. 1. The protein concentration determined using the Bradford protein assay is 2 µg/µl.

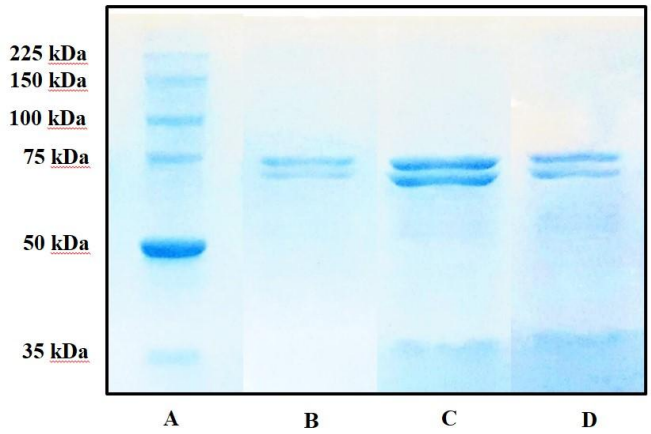

Fig. 1 SDS-PAGE visualization shows the DBL2β-PfEMP1 recombinant protein of ~72 kDa: A – marker, B - protein elusion using 30 mM imidazole, C - protein elusion using 60 mM imidazole, and D - protein elusion using 100 mM imidazole (The authors)

3.2. IgM, IgG, and CD4+ Concentrations

IgM, IgG, and CD4+ concentrations for all groups were measured using ELISA, as shown in Fig. 2. IgM concentration increased with increasing protein dose and injection frequency. A similar trend was observed for CD4+ concentration. However, IgG concentration demonstrated a different response; its concentration was lower after the second injection in all treatment groups, and the highest response of 150 µg dose was observed after the first injection. However, overall, the highest IgM, IgG, and CD4+ concentrations were observed at the 200-µg dose after the third injection.

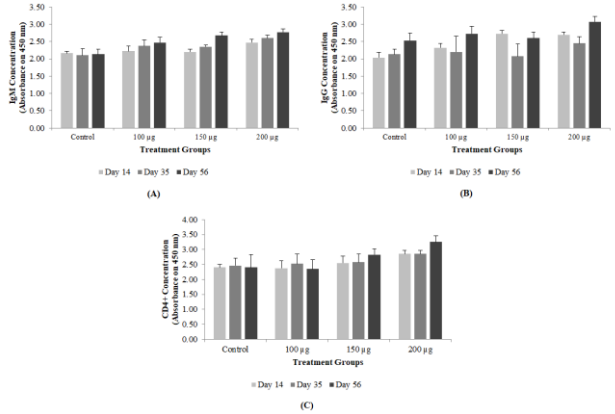

Fig. 2 Levels of (A) IgM, (B) IgG, and (C) CD4+ in the control and treatment groups. Light gray bars show the level on Day 14, gray bars show the level on day 35, and black bars show the level on Day 56 (The authors)

The data on IgM, IgG, and CD4+ concentrations were statistically analyzed using a mixed-method ANOVA test. The results showed significant differences between the control and treatment groups with p=0.001, 0.033, and 0.003 for IgM, IgG, and CD4+, respectively (Table 1). Further analysis was conducted using the Bonferroni post-hoc test. For the IgM concentration, a significant difference was observed between the control and 150-µg dose groups, control and 200-µg dose groups, and 100-µg and 200-µg dose groups. The IgG concentration showed a significant difference only between the control and 200-µg dose groups. Moreover, the CD4+ concentration demonstrated a significant difference between the control and 200-µg dose groups and 100-µg and 200-µg dose groups.

Table 1 Results of statistical analysis using mixed-method ANOVA followed by the Bonferroni post-hoc test (The authors)

	Control	100 µg	150 µg	200 µg	ANOVA test (p)
IgM Concentration					
Control		0.096	0.031*	0.000*	
100 µg	0.096		1.000	0.029*	
150 µg	0.031*	1.000		0.096	
200 µg	0.000*	0.029*	0.096		0.001
IgG Concentration					
Control		1.000	0.865	0.034*	
100 µg	1.000		1.000	0.225	
150 µg	0.865	1.000		0.424	
200 µg	0.034*	0.225	0.424		0.033
CD4+ Concentration					
Control		1.000	0.599	0.006*	
100 µg	1.000		1.000	0.010*	
150 µg	0.599	1.000		0.094	
200 µg	0.006*	0.010*	0.094		0.033

* A significant difference ($p<0.05$)

4. Discussion

The development of a malaria vaccine is challenging due to the low efficacy of acquiring malaria protection [25]. The DBL2β-PfEMP1 recombinant protein is a vaccine candidate currently being developed because of its ability to generate specific IgG antibodies and CD4+ lymphocyte T cells in Wistar rats [12]. Determining the immune response generated by several doses of DBL2β-PfEMP1 recombinant protein is vital in developing a malaria vaccine. Using serial doses of 100, 150, and 200 µg, which are given consecutively in three-time injections, this study showed that the highest response in inducing humoral and cellular immunity was 200 µg. The result is supported by post-hoc statistical analysis, which showed that the dose of 200 µg consistently differs from the control group for IgM, IgG, and CD4+ concentrations. Increased doses of protein generated higher IgM, IgG, and CD4+ concentrations, along with the injection frequency, except for the second injection for IgG concentration (Fig. 2).

The increased IgM concentration after the second and third injections indicated a higher specificity and affinity toward the recombinant protein during the secondary antibody response. A previous study reported that IgM is an essential functional antibody that significantly contributes to naturally acquired protective immunity against malaria. This antibody targets merozoites, is a substantial component of malaria even among those with many prior exposures, and can persist following an acute infection. Furthermore, there is a suggestion that there is induction and maintenance of long-lived IgM-secreting cells and/or memory IgM B cells following clinical malaria [17]. The expanded memory B cell clones evoke the response to enact an indispensable role in maintaining long-term protective humoral immunity against *P. falciparum*, as reported in [26, 27].

The highest increase in IgG concentration was observed after the third injection, especially at the 100 and 200 µg doses. However, the second injection demonstrated a different result. This study used a 3-week interval between injections to determine the immune response time. In malaria infection, IgG antibodies are an important component of acquired immunity. Although studies have found that total IgG against malaria antigens is a poor predictor of immunity, each of the four IgG subclasses (IgG1-IgG4) played different roles in acquiring naturally acquired immunity to malaria [28]. Moreover, the examination time could be a factor in the antibody concentration in sera because there are short-lived antibody-secreting cells for the IgG subclass (approximately 28-day half-life for IgG3 and 36-day half-life for IgG1) [28]. The increased IgG antibodies observed along with the injection frequency in all doses of treatment groups implied immune response stimulation by the DBL2β-PfEMP1 recombinant protein by activating the antigen-presenting cell (APC). A high IgG concentration is an important indicator of malaria vaccine development because it inhibits merozoite invasion, cytoadherence, and rosette formation [6, 10].

Studies have revealed that T cells also play a major role in the acquisition and maintenance of protective immunity against malaria infection. Both animal models and humans indicate the CD4+ cell subsets' important role in conferring protection against malaria. Previously, this cellular immune response was commonly known due to its important role in antibody production elicitation. However, recent studies have indicated the role of CD4+ cells independently of B cells in parasite resolution. Developing long-term memory B cells that produce antibodies requires CD4+ cells, particularly T-follicular helper (Tfh) cells and Th1 cells [11, 29]. Fig. 2 shows the increase of CD4+ cells along with the injection frequency, especially in the 200-µg group. CD4+ maintenance is vital to prevent the chronicity of infection [30]. Regardless of the inflammatory condition often observed in acute malaria infection, CD4+ cells are usually at a suboptimal level and thus unable to form and induce immunological memory. Hence, a recombinant protein that can induce robust production of CD4+ cells could be a potential vaccine candidate [31].

5. Conclusion

The study demonstrated an increase in IgM, IgG, and CD4+ concentrations after consecutive injections of the DBL2β-PfEMP1 recombinant protein, and a dose of 200 µg induced the highest IgM, IgG, and CD4+ concentrations. Several studies on malaria vaccine candidates based on recombinant *P. falciparum* blood stage protein used a dose range of 50–160 µg to elicit humoral and cellular immune responses. These findings implied that a dose of 200 µg is an effective dose of DBL2β-PfEMP1 recombinant protein to induce humoral and cellular immune responses and could be used as a malaria vaccine. Further studies on the safety of the DBL2β-PfEMP1 recombinant protein to vital organs and the toxicity effect should be conducted to develop it as a malaria vaccine.

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