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Vitamin A Modulation toward IL-12, IFN-γ Production and Macrophage Activity in Malaria Disease

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Abstract. Malaria is a global disease and kills over 30 % to 40 % of children in endemic areas or developing countries, including Indonesia. Healing rates of malaria is affected by micronutrient body consumption such as vitamin A. Vitamin A plays an important role in enhancing the immune system. Vitamin A deficiency may weaken the body's resistance to infectious diseases. This study aims to examine potent dose of vitamin A that induces an immune response as an immunomodulator using experimental mice infected with *Plasmodium berghei* through cellular immunity parameters analysis. This research was an experimental laboratory, designed as Post Test-Only Control Group Design. Experimental male Swiss Strain mice, which were eight weeks old, healthy and weighed 28.80 g to 31.20 g, were treated with standard feeding and drinking through *ad libitum*. A total of 24 mice which had been adapted for a week were randomly divided into four groups: K (control); P1 (solvent of vitamin A); P2 (treated with vitamin A and doses of 3 000 IU) and P3 (treated with vitamin A and doses of 6 000 IU) for examination of the cellular immune response. Oral administration of vitamin A was given one week before and 1 h after infection. All mice were infected with $10^7 \cdot ml^{-1}$ *Plasmodium berghei* intraperitoneally on 15 d. On the 25 d, all mice were killed and examined for the level of IL-12, IFN-γ levels and macrophage phagocytosis index. Test results were analyzed by ANOVA with significance limit < 0.05. Result showed that vitamin A improved cellular immune response characterized by increasing levels of IL-12, IFN-γ and macrophage phagocytosis index. This study also showed that vitamin A was potential to be developed as a cellular immunomodulator to fight against malaria with the optimal dose is 6 000 IU.

Keywords: IL-12, Immunomodulator Macrophage activation, INF-γ, malaria, vitamin A.

INTRODUCTION

There are 198×10^6 cases and 584×10^3 deaths caused by malaria and more than 50 % are in Africa [1] with the majority of sufferers are children [2]. Malaria may decrease productivity and health status of the population and then inhibit social and economic development [3].

Immunity against malaria is too complex because it involves almost whole components of the immune system both specific and non-specific. Non-specific immune response or natural first effectors provides resistance to infection mainly by skin, macrophages, neutrophils, cytokines and spleen [4[.

To keep the immune system work optimally, can be done by consuming micronutrients. One micronutrient developed to enhance the immune system is Vitamin A. Research in several developing countries also showed correlation of differences amount of Vitamin A and infection status in children. Vitamin A also plays a role in immune function [5], growth, development, reproduction, [6] as well as the stabilization of cell membranes [7].

Research on pre-school children showed there is a correlation between vitamin A level in plasma and *Plasmodium falciparum* parasitemia level [8–11]. Supplementation of vitamin A in pre-school children had lowered visitation of malaria patient to the health center over 38 %. According to Pittman [12] and Ross [13], vitamin A in

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the body acts as an immunomodulator with direct effect on cells of the immune system, activates and stimulates macrophages, monocytes, antibodies and cellular immunity of T cells. It induces immune cell activities to kill intracellular protozoa in two ways: i) The cytokines produced by T lymphocytes, especially interferon γ (IFN-γ) activates macrophages and increases phagocytosis; ii) cytotoxic T lymphocytes damage the membranes of infected cells by intracellular microorganisms (14). Vitamin A is able to regulate the differentiation of T cells intra-thymus in *in vivo* condition. Supplementation of vitamin A in the mice with vitamin A deficiency may increase serum complement and titer of Immunoglobulin (Ig). Increased Ig production has been estimated because vitamin A plays particular roles in increasing the synthesis of Interleukin 4 (IL-4) by T helper cells [15].

Based on that reason, the research aimed to determine i) the correlation between vitamin A supplement intake and level of IL-12 and IFN-γ and ii) the optimum dose of vitamin A as the most potent immunomodulators in the immune response to malaria. The greater the level of IL-12, the greater IFN-γ acting as a modulator in activated macrophages. This shows that the occurrence of immunomodulators on the immune response of malaria assessed: i) low level of parasitemia; ii) increased level of IL-12; and iii) increased phagocytosis index using macrophages.

MATERIAL AND METHOD

This research was an experimental laboratory research with The Post Test-Only Control Group Design using experimental animals as objects of research [16]. The number of samples was determined by the Federer's formula for experimental research, using 24 mice which were divided into four groups. This research was conducted in the Laboratory of Biochemistry, Biology, Universitas Negeri Semarang; Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM) and Laboratory of Iodine Deficiency Disorder (*Laboratorium* GAKI - *Gangguan Akibat Kekurangan Iodium*) Faculty of Medicine, Universitas Diponegoro.

Samples

Purposive samples took from male Swiss strains mice with the same genetic trait, health, activity and normal behavior, age eight weeks, weighing from 28.80 g to 31.20 g. Mice were grouped randomly and weighed before and after the transfer in order to avoid bias by factors of age and weight variation. Swiss strain was used in this study because it had been reported to induce cellular immune response when mice were inoculated with *Plasmodium berghei*.

Treatment

Experiments were carried out with the completely randomized design. Mice were divided into four groups adapted for seven days and treated in the laboratory with adequately housed for, fed standard and drink *ad libitum* until the 14 d. Three groups, Group 2, Group 3 and Group 4 were given with vitamin A for a week. After Vitamin A treatment, whole groups were infected intraperitoneally with *Plasmodium berghei* at the 15 d and treated back with vitamin A an hour after infection, four groups treatment consisted of Group 1 (C): Control (10^7 cell · mL^{-1} *Plasmodium berghei*); Group 2 (P1): 10^7 cell · mL^{-1} *Plasmodium berghei* with vegetable oil and olive oil 1 cc; and Group 3 (P2): 10^7 cell · mL^{-1} *Plasmodium berghei* and vitamin A with dose of 3 000 IU; and Group 4 (P3): 10^7 cell · mL^{-1} *Plasmodium berghei* and vitamin A with dose of 6 000 IU. After 10 d, all mice were killed to check Interleukin 12, Interferon γ and macrophage index using kit method research.

Materials

Plasmodium berghei, a hemoprotozoan causing malaria in rodents which obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Gajah Mada (UGM), cultured in laboratory of Animal Physiology of Swiss mice in Universitas Negeri Semarang. The inoculum was prepared by diluting a number of blood donors with parasitemia 30 to 40 % in RPMI 1640.

Mice were obtained from the Laboratory of Parasitology, Faculty of Medicine, UGM, and maintained in an iron cage measuring 50 cm × 30 cm × 20 cm, with six mice in each cage, were fed with pellets BR2 and were given drinking water. Vitamin A used in this research contain Vitamin A acetate; each tablet contains 6 000 IU. The solvent used as vegetable oil was olive oil.

Samples were taken from male Swiss mice in the form of peritoneal exudate cells (PEC). Feeding was obtained from the Food and Nutrition PAU UGM. Reagents needed were: Roswell Park Memorial Institute (RPMI), complete, solution Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS) 10 %, 70 % alcohol, penicillin and streptomycin, 3 % acetic acid, Latex beads, absolute methanol, Phosphate Buffered Saline (PBS), Griess reagent (chromogenic reagent), Canada Balsam, 20 % Giemsa solution, sterile distilled water, BHI media, physiological saline, and NH_4Cl.

IL-12, IFN-γ and Macrophage Measurement

This research used ELISA kit to measure both IL-12 and IFN-γ. The blood was obtained from orbital sinus using hematocrit pipe and collected in the tube that had been smeared with EDTA. The blood was centrifuged at 10 000 rpm for 10 min (1 rpm = 1/60 Hz). The serum was divided into the different tube using micropipettes. IL-12 measurement has been done with The IL-12p70 Mouse ELISA Kit and the procedure has followed the instruction from the fabric. Meanwhile, INF γ was measured from the serum that had been collected before and followed the procedure of IFN-γ Mouse ELISA Kit instruction from the fabric.

After the blood had collected, mice were killed using *petroleum ether* and then 10 mL RPMI was injected through peritoneum narrow, allowed to stand for three minutes and retook with a syringe. The fluid from peritoneum narrow has been centrifuged at 1 200 rpm for 10 min. The cell was measured using hemocytometer and suspended until 2.5×10^6 cell · mL^{-1} then incubated in CO_2 5 % incubator for 24 h. After cultured, macrophage was washed twice using RPIM and mixed with 200 µL *latex beads* then incubated at 37 °C for 60 min. Incubated macrophage washed with PBS and got dried. The macrophage was then stained using Giemsa 20 % for 30 min and washed with distilled water. Finally, the number of macrophages that phagocytes the latex and the number of latex in the macrophages were calculated.

Statistical analysis

Before hypothesis test, data was entered into computer files and cleaned and then analyzed descriptively. The data was tested using Kolmogorov-Smirnov and showed normal distribution. Data of IL-12 and IFN-γ level and macrophage phagocytosis index were tested using One-way ANOVA and followed by Least Significant Difference (LSD)/Least Significant Difference (LSD). All test statistical analysis was conducted with SPSS 13 [17]. The data was significant at $p < 0.05$.

RESULTS

This study used vitamin A doses which equivalent with recommended dose for children patients with chickenpox disease. During the trial period, the general state of the mice weight was observed (Table 1). During the experiment, mice showed no signs of hypervitaminosis A, such as hair loss. This means that doses of vitamin A given to mice in the amount of 3 000 IU and 6 000 IU did not cause poisoning symptoms.

TABLE 1. Mice body weight before and after treatment

Group	Average Weight		Differences
	before treatment (g)	10 d after treatment (g)	
Control	30.00	19.72	10.28[a]
Solvent	30.10	19.81	10.29[a]
Vit A 3 000 IU	30.60	23.30	7.30b[b]
Vit A 6 000 IU	30.62	25.22	5.40[b]

[a,b,c] Different letters indicate significant differences at level 5 %

Mice Group 1 and Group 2 appear to be inactive on 6 d to 8 d post-infection. Groups of mice were given vitamin A does not appear amendment until mice were killed on 10 d post-infected.

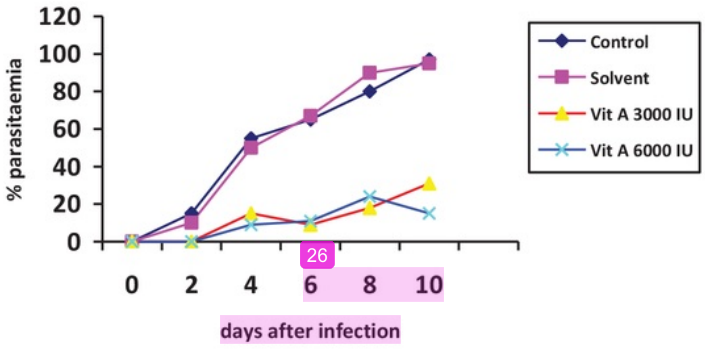

FIGURE 1. Parasitaemia index of *Plasmodium berghei* in the treatment and non-treatment groups

Parasitaemia (Fig. 1) in group 1 and group 2 began to be detected in 2 d post-infection, followed by a rapid increase in parasitemia and led to death. The deaths occurred in the group of mice on 8 d to 10 d post infection with parasitemia between 70 % to 90 %, while Groups 3 and 4, the new parasite was detected on 4 d post-infection. In the third group there was an increase of 9 % parasitaemia on 6 d to 15 % at 8 d and to 31 % on the 10 d whereas the second group of 11 % on 6 d to 24 % on 8 d and to 15 % at 10 d.

TABLE 2. Levels of IL-12 in control mice and exposed to *Plasmodium berghei*

Group	IL-12 (pg · mL^{-1})*
1	(1.9 567 ± 0.32 377)^{a}
2	(1.9 550 ± 0.35 820)^{a}
3	(14.1 550 ± 2.68 967)^{b}
4	(57.7 800 ± 5.86 866)^{c}

^{a,b,c} Different letters indicate significant differences at level 5 %
*mean ± sd

Levels of IL-12

The highest average level of IL-12 was found in group 4, achieving (57.7 800 ± 5.86 866) pg · mL^{-1}, while the lowest average was found in group 1, the group of mice given solvent vitamin A [(1.9 550 ± 0.35 820) pg · mL^{-1}] (Table 2). The test results least significant difference (LSD) showed that there was no significant difference between group 1 and group 2 but there was significant difference between group 3 and 4.

TABLE 3. Levels of IFN -γ and in control mice and exposed to *Plasmodium berghei*

Group	IFN-γ (pg · mL^{-1})*
1	(6.2 093 ± 18.17 774)^{a}
2	(6.2 020 ± 12.87 154)^{a}
3	(6.6 251 ± 4.43 345)^{b}
4	(7.8 775 ± 21.96 332)^{c}

^{a,b,c} Different letters indicate significant differences at level 5 %
*mean ± sd

Levels of IFN-γ

The highest level of IFN-γ was found in group 4, achieving (7.8 775 ± 21.96 332) pg · mL^{-1} while the lowest average was found in group 2 is ± 6.2 020 ± 12.87 l3 pg · mL^{-1} (Table 3).

The test results of least significant difference (LSD) showed that there was no significant difference between group 1 to group 2 but there was significant difference between group 3 and group 4.

TABLE 4. Levels of macrophages phagocytic index in control mice which were exposed to *Plasmodium berghei*

Group	Macrophages Phagocytic Index (%)*
1	$(1.1\,443 \pm 0.3\,364)^{a}$
2	$(1.1\,535 \pm 0.2\,737)^{a}$
3	$(1.5\,225 \pm 0.2\,411)^{b}$
4	$(3.9\,168 \pm 0.2\,272)^{c}$

[a,b,c] Different letters indicate significant difference at level 5 %
*mean ± sd

Macrophages Phagocytosis Ability

The highest macrophage phagocytosis index was in group 4, reaching [(3.91 683 ± 0.227 190) %], while the lowest average macrophage phagocytosis index was found in group 1, approximately (1.14 433 ± 0.336 419) % (Table 3)

The test results least significant differen3 (LSD) showed that there was no significant difference between groups 1 to group 2. The test results showed no significant difference between group 1 with group 3 and group 4. The test results also showed significant difference between groups 3 with group 4. Low values of macrophage phagocytic index possible caused by limited treatment duration. At the 8 d, all mice in control group was died before finished. So, it needed to kill all mice in the same time to conduct valid measurement and representative data. It is possible the percentage of macrophage phagocytic index will increase after 8 d.

DISCUSSION

Based on the study, oral admin25ation of vitamin A has increased nu19r of IL-12 and IFN-γ levels. Statistically, significant difference of IL-12 and IFN-γ levels can be observed in group 1 with group 3 and group 4. 24anwhile, in group 1 and group 2, there was no significant difference. It shows that vitamin solvent did not affect IL-12 and IFN-γ levels.

Observations were conducted to determine vitamin A effect in the mice infected with *Plasmodium berghei*. Vitamin A only worked in general terms, such 20in the mechanism of proliferation and differentiation of immune cells. Even in the low compound of vitamin A in the body, immune system is running optimally [18]. The addition of vitamin A only has little effect. However, various studies indicate that specific vitamin A has a function in the immune response, especially in macrophage activities. Low intake of vitamin A also improves cells ability to proliferate [19].

Based on the research data, IL-12 level in group 3 and group 4 were higher than group 1 and group 2. This shows the possibility of vitamin A to modulate the immune system. Increasing IL-12 number triggered by the activation of macrophages into Dendritic Cell (DC) in an effort to fight against the antigen [20]. Vitamin A in the form of provitamin carotenoids and other compounds are easier to be absorbed. Pro-vitamin A would be absorbed by epitel cell in the intestine and converted into retinaldehyde in the cytoplasm [21]. In the cytoplasm, Retinaldehyde will be changed into retinoic acid that will be bonded to the active site of Retinoids A Receptor (RAR) and Retinoid X Receptor (RXR). Retinoic Acid Complex-RAR/ RXR heterodimer is component to activate cell DNA and also as a transcription trigger of acute phase response proteins such as IL-12 and INF α [22].

The high number of IL-12 will stimulate T cell proliferation becomes Th cell and influence NK and Th1 and Th2 cell to secrete IFN-γ [23]. The results showed that IFN-γ levels also increased high enough, the highest production of it has been found in the mice which given 6 000 IU with vitamin A. IFN-γ which secreted will reactivate macrophages. Activation of macrophages into DC boost phagocytosis activity in term of against the antigen.

Vitamin A plays a role as a mediator of immunity, especially for timulin. It is a non-peptide hormone produced by thymus epithelial cells and vitamin A requires as a coenzyme in biological activity. The peptide helps the

maturation of lymphocytes T, cytotoxicity and the production of IL-12. Timulin activity in vitro both in experimental animals and humans is highly dependent on the levels of plasma vitamin.

Vitamin A also plays a role in the activity of cytokines, vitamin A deficiency is a trigger in declining of Th1 and Th2 number also increasing parasite amount [19]. In the state of vitamin A deficiency, Timulin becomes inactivated, so that the function of differentiation, proliferation and maturation lymphocyte T also decreases. Lymphocyte responses such as delayed hypersensitivity and cytotoxic activity also decreased during vitamin A deficiency and improved after supplementation of vitamin A. That information was in line with the research result, indicating that the mice without vitamin A showed high amount of parasite (Fig. 1).

Vitamin A affects membrane stability, especially at the level of the cytoskeleton. Vitamin A effects on the membrane can be seen by increasing phagocytosis ability, oxygen consumption and bactericidal activity in phagocytic cells and modification of the receptor on the cell surface Con-A limfoid. Vitamin A is a major regulator of intracellular immunity in vitro and in vivo. Giving vitamin A has increased the titer of Immunoglobulin G that estimated by synthesis of IL-4 by Th cells. The natural immune system function is affected by the levels of vitamin A. In vitro, not only affect neutrophil granulocyte chemotactic activity but also on nuclear Polymorphism (PMN). In the in vivo, the activity of NK cell, macrophage, and neutrophil deprecated as well as the number of granulocytes which influenced by decreasing levels of vitamin A. In specific immunity, vitamin A deficiency causes a decrease in the number and function of lymphocytes, macrophages, T helper lymphocyte ratio decrease / Th ($CD4^+$): cytotoxic T lymphocytes ($CD8^+$), decrease in the number of lymphocytes T $CD8^+$ $CD73^+$, which are precursors of cytotoxic T lymphocytes, B lymphocytes decrease antibody response, and decreased the cytokine [23, 24].

Cells that play an important role in the body's defense against *Plasmodium berghei* in mice are NK cells (Natural Killer) and macrophages [24]. In this phase, the rapid growth of microorganisms in the liver and spleen were growth will settle under the influence of activated macrophages. Activation of macrophages is the effect of the cytokine IFN-γ is in the early phase of infection is mainly produced by NK cells [25].

This study assesses the effect of vitamin A on one indicator of activation of macrophages, the phagocytic ability, which is assessed by counting a number of macrophages had digested latex in the state of *Plasmodium berghei* infection. In this study, the dose of vitamin A in mice infected *Plasmodium berghei* is able to activate the macrophages and characterized by the high ability of macrophage phagocytosis compared with control mice.

CONCLUSION

Giving vitamin A per oral solution can increase innate immunity response and acquired immunity in Swiss strain mice which are infected with *Plasmodium berghei*. This is indicated by the occurrence of elevated levels of IL-12, IFN-γ and phagocytic index. The optimal dose, which is 6 000 IU, causes significant impact on the declined index parasitaemia. It should also continue to see increased activity of molecular cell and organ damage as a result of high doses of vitamin A.

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