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Evaluation of the Adjuvant Activity of Propranolol, a Beta-Adrenergic Receptor Antagonist, on Efficacy of a Malaria Vaccine Model in BALB/c Mice

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ABSTRACT

We have previously shown the adjuvant activity of propranolol (PRP) (a beta-adrenergic receptor antagonist) using a vaccine model for *Salmonella typhimurium*. In this study PRP was used as an adjuvant in combination with *Plasmodium berghei* (*P. berghei*) whole blood stage (PWBS) antigens.

BALB/c mice were immunized three times with a 2-week interval, either PWBS vaccine alone or in combination with the adjuvant alum or propranolol. The control group received phosphate buffered saline. Evaluation of the cellular and humoral immunity was performed by measurement of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, lymphocyte proliferation, total IgG and IgG2a in the control and immunized groups. Furthermore, Clinical evaluations were carried out by analyze survival rate and parasitemia of the mice.

Our results showed that the mice immunized with propranolol induced higher levels of antibody, IFN-γ and TNF-α as well as stronger lymphocyte proliferative responses compared with other groups. This resulted in improved protective immunity against *Plasmodium berghei*.

Administration of the PRP as an adjuvant in combination with the PWBS Antigen vaccine can shift the immune responses to a T helper1 pattern and enhance the protective immunity.

Keywords: Adjuvant; Beta-adrenergic receptor; Malaria; *Plasmodium berghei;* Propranolol; Vaccine

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INTRODUCTION

Malaria is a major global health problem with 3.2 Billion people at risk, 500 million clinical infections

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that results nearly in 1-2.5 Million deaths annually.[1] Because of the increasing resistance to insecticides and antimalarial drugs, an affordable and effective vaccine remains a high priority.[2,3]

However, in spite of intensive efforts during the past decades, only a few blood-stage vaccines have been tested in preclinical and clinical trials.[4] This type of vaccines have shown poor efficacy. It seems that the reason for this failure is mainly due to lack of an appropriate adjuvant.

The vast majority of large number of adjuvants examined in animal models, are not safe for human use because of their toxicity.[5] Alum (aluminum-based mineral salt) is the only vaccine adjuvant that is approved by the United States Food and Drug Administration (FDA).[5] The oil-in-water emulsions MF59 and AS03 and the monophosphoryl Lipid A formulated in alum (AS04) are other adjuvants that have been approved by the European Medicines Agency as vaccine adjuvants.[5-7] However, alum remains the most important adjuvant approved worldwide for human use.[8] Alum exists in many licensed vaccines.[8,9] However, the adjuvant activity of alum has some limitations. The major limitations is alum's inability to stimulate cell-mediated T helper1 (Th1) responses that are required to control most intracellular pathogens.[8,10] Therefore, alum is not an appropriate adjuvant for anti-malaria vaccines because Th1 immune responses play an important role in immunity against plasmodium species especially in the acute phase of the disease.[11-16]

One probable mechanism to modulate immune responses is via affecting sympathetic nervous system. Catecholamines that are secreted from sympathetic nerves or adrenal glands and also antigen presenting cells (APCs)-derived catecholamines can modulate immune reactions.[17-21] Catecholamines can deviate immune responses toward T helper 2 (Th2) profile and suppress Th1 immune responses. Stimulation of beta-adrenergic receptors (β-ARs), especially β2-ARs, on the surface of APCs and T lymphocytes are responsible for such an effect.[22,23] We have previously shown the adjuvant activity of propranolol (PRP) (a beta-adrenergic receptor antagonist) using a vaccine model for *Salmonella typhimurium*.[24] In the current study, we used PRP, a pan β-blocker as an adjuvant in combination with *Plasmodium berghei* (*P. berghei*) whole blood stage antigens (PWBSA) as a vaccine model. Alum was used in this study as a well-known adjuvant to be compared with PRP.

MATERIALS AND METHODS

Female BALB/c mice (6-8 week old) were purchased from the Razi Vaccine and Serum Research Institute of Iran. All studies were performed according to the Animal Care and Use Protocol of Urmia University of Medical Sciences (Urmia, Iran). *Plasmodium berghei ANKA strain* was a gift from Dr. Peter F. Billingsley (Zoology Department Aberdeen University, United Kingdom). Parasites were maintained in our laboratory by alternating passage of parasites through *Anopheles stephensi* mosquitoes and BALB/c mice.

Preparation of PWBSA

Infected blood, having 20% infection, was collected in a heparin tube by cardiac puncture and washed in RPMI1640 medium then resuspended in Phosphate buffered saline (PBS). Plasmodium-infected Red blood cells (RBCs) were separated and harvested using a 53% Histodenze (Sigma) gradient as previously described[25] and centrifuged at 1300g for 30 minutes at 4 °C. The infected erythrocytes were washed 2 times with PBS buffer and lysed with ammonium chloride (0.9%). Cell free parasite pellet was washed twice by PBS and then was homogenized and subsequently was autoclaved at 15 lbs for 20 min. Protein concentration was determined by Bradford method. Antigen preparations were kept in small aliquots at -20 ◦C until use.

Immunization Protocol

Mice were divided into four groups and immunized subcutaneously (s.c.) for a total of three times, at days 0, 14 and 28. To prepare the PRP solution, 6 mg/kg of PRP (Sigma, Germany) was dissolved in 50 µl of PBS. For preparation of the antigen, 20 µg of PWBSA protein was suspended in 50 µl of PBS. PRP–Vac group received the protein suspended in 100 µl of PBS plus PRP (6 mg/ kg), and then dissolved in 50 µL of PBS. Al-Vac group received the protein suspended in 100 µl of PBS that was absorbed in 50 µL of alum (aluminum phosphate gel, Sigma, Germany). Vac group received the protein suspended in 150 µL of PBS and as negative control; mice were inoculated with 150 µL of PBS.

Lymphocyte Proliferation Assay

Two weeks after the last immunization, the lymphocyte proliferation rate was measured in five mice from each group using an MTT (3[4, 5-dimethylthiazol-2-µl]-2, 5-diphenyltetrazolium

bromide; thiazolyl-blue, Sigma, Germany) dye assay. The spleens of the mice were removed under sterile conditions and single-cell suspensions were prepared in phenol red-free RPMI 1640 medium. RBCs were lysed using 0.75% ammonium chloride in Tris buffer (0.02%, pH 7.2). The cell concentration was adjusted to 1×10^6 cells/ml in phenol red-free RPMI 1640 that was supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, and 25 mM HEPES. One hundred microliters of diluted cell suspensions were dispensed into 96-well flat-bottom culture plates. Mitogen phytohemagglutinin-A (Gibco-BRL) at a final concentration of 5 µg/ml (positive control) or 20 µl of the PWBSA suspension was added to each well and the volume was adjusted to 0.2 ml. After incubating for 48 hours at 37 ºC in 5% CO_2, cell proliferation was determined using an MTT assay.[26]

Cytokine Assays

Two weeks after the last immunization, mice sera from each group were collected and tumor necrosis factor alpha (TNF-α) were detected by using the commercial kit Mouse TNF-α Platinum ELISA (eBioscience) according to the manufacturer's instructions. For IFN-γ, Spleen cells were removed as above and after 72 h of culture the level of cytokine IFN-γ in the culture supernatants were measured by commercial ELISA kit (ELIZAPRO kit for Mouse IFN-γ , MabTech).

Determination of Total IgG and IgG2a Levels

Two weeks after the last immunization, the levels of specific total IgG and IgG2a antibodies were measured in the sera of five mice from each group by ELISA. Briefly, 200 µl of PWBSA, as parasite antigen (with the concentration of 20 µg protein/ml) in coating buffer (0.1 M carbonate, pH 9.5) was added to each well. Coated plates were incubated at 4°C overnight, washed with PBS with 0.05% Tween 20 (PBST) three times, and blocked with 5% bovine serum albumin in PBST for 2 hours at 37°C. After washing the plates with PBST, different dilutions of sera were addedand plates were incubated at 37°C for 2 hours. After washing three times with PBST, the plates were incubated with HRP-conjugated rabbit anti-mouse IgG (RAY BioTech) or IgG2a (AbDSerotec, USA). After washing three times with PBST, the reaction was developed by adding 200 µl of TMB/H_2O_2 substrate. The reaction was terminated by the addition of 50 µl of 2.0 N H_2SO_4 and the absorbance was read at 450 nm wavelength.

Survival Rate and Parasitemia

Two weeks after the last immunization, 7 mice from each group were challenged by an intraperitoneal inoculation of 100 µl *P. berghei* infected RBCs (average 25% parasitemia). The survival rates were monitored for 23 days, and the number of dead mice were recorded every day. Furthermore, murine parasitemia was assessed on days 5, 7 and 9 by microscopic examination of Giemsa-stained thin smears of tail blood.

Statistical Analysis

Antibody assay, proliferation assay and cytokine results were analyzed by analysis of variance (ANOVA) followed by Tukey test. The survival rate was measured using Kaplan–Meier analysis and the log rank test. Malaria parasitemia was highly skewed to higher values and was transformed by $\log_{10}$ to normalize the distribution [27] and then the analyses were performed using ANOVA followed by Tukey test. $P<0.05$ was considered statistically significant.

RESULTS

Lymphocyte Proliferation

To assess cell-mediated immunity in immunized mice, MTT test was performed. As it is shown in figure 1, there were significant differences in lymphocyte proliferation of the mice in PRP-Vac group compared to those of the mice in Al-Vac, Vac and control groups. There were no significant differences in lymphocyte proliferation among other groups.

Cytokine Assays

As it is shown in figure 2a, the mice of PRP-Vac group, produced larger amounts of IFN-γ than those of the mice of Al-Vac, Vac and PBS .

Figure2b shows the levels of TNF-α in sera of the mice two weeks after the last vaccination. The sera of the mice in PRP-Vac group contained significantly higher levels of TNF-α compared to those of the mice in Al-Vac, Vac and control groups ($p<0.0001$) The level of TNF-α in sera of the mice in Vac group was more than those of Al-Vac and control groups but the difference was not statistically significant. There was no significant differences between the levels of TNF-α of the mice in Al-Vac and control groups.

Antibody Responses

Serum samples obtained two weeks after the last immunization were analyzed for the parasite specific antibodies by ELISA. As it is shown in figure 3a, the mice in the PRP-Vac group produced significantly higher anti-*P. berghei* IgG2a comparing to the mice in control group . The level of anti-*P.berghei* IgG2a in sera of the mice in Vac group was more than those of Al-Vac and control groups but the difference was not statistically significant. The mice of Al-Vac group produced levels of anti-*P. berghei* IgG2a comparing to the mice in control group but again the difference was not statistically significant.

As it is shown in figure 3b, the level of anti-*P. berghei* total IgG in sera of the mice in PRP-Vac group was significantly more than that of control group. The production of anti-*P.berghei* total IgG by the mice in the PRP-Vac group was higher than that of the mice in Vac group but less than that of the mice in Al-Vac group, however, the differences were not statistically significant. The mice in Al-Vac group produced significantly higher anti-*P. berghei* total IgG than those of the mice in Vac and control groups. The level of anti-*P.berghei* total IgG in sera of the mice in Vac group was more than that of control group but the difference was not statistically significant.

Parasite Challenge

To determine the efficacy of vaccination with the various adjuvants in conferring protective immunity, groups of BALB/c mice, immunized as described above, were challenged with *P.berghei* two weeks after the inoculation.

As it is shown in figure 4, the mean survival rate of mice in control group was significantly lower than those of mice in Vac, Al-Vac and PRP-Vac groups . Mice of PRP-Vac group showed more mean survival rates than those of mice in Al-Vac and Vac groups, however, the differences were not statistically significant. The mean survival rate of the mice in Al-Vac group was more than that of Vac group but again, the difference was not statistically significant.

Furthermore, parasitemia of the mice were determined in the fifth, seventh and ninth days after the parasite challenge (Figure 5). On the fifth day after the parasite challenge, there was no parasitemia in the mice of Vac, Al-Vac and PRP groups. On the seventh day after the parasite challenge, there was no parasitemia in the mice of Al-Vac and PRP-Vac groups and the mean parasitemia of the mice of control and Vac groups were significantly more than those of the mice in Al-Vac and PRP-Vac groups .

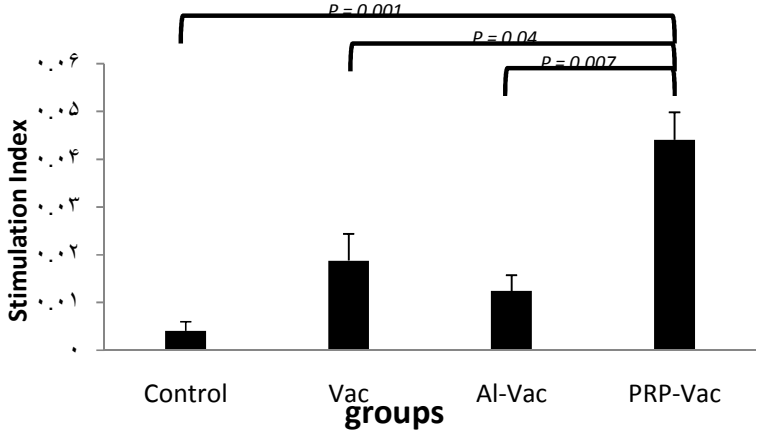

Figure 1. Effect of PRP on lymphocyte proliferation.

Two weeks after the last immunization, splenocytes from the mice immunized with adjuvant were stimulated with PWBSA in vitro and lymphocyte proliferation was evaluated using the MTT method and then compared in control, Vac (PWBSA vaccine alone), Al-Vac (alum in combination with the PWBSA vaccine) and PRP-Vac (PRP in combination with the PWBSA vaccine) groups. The mice in PRP-Vac group showed significantly higher lymphocyte proliferations comparing to those of the mice in Al-Vac, Vac and control groups. Stimulation indices (SI) were determined and expressed as differences between the absorbance of treated and untreated wells. The values are mean ±SE (n= 5 mice per group).

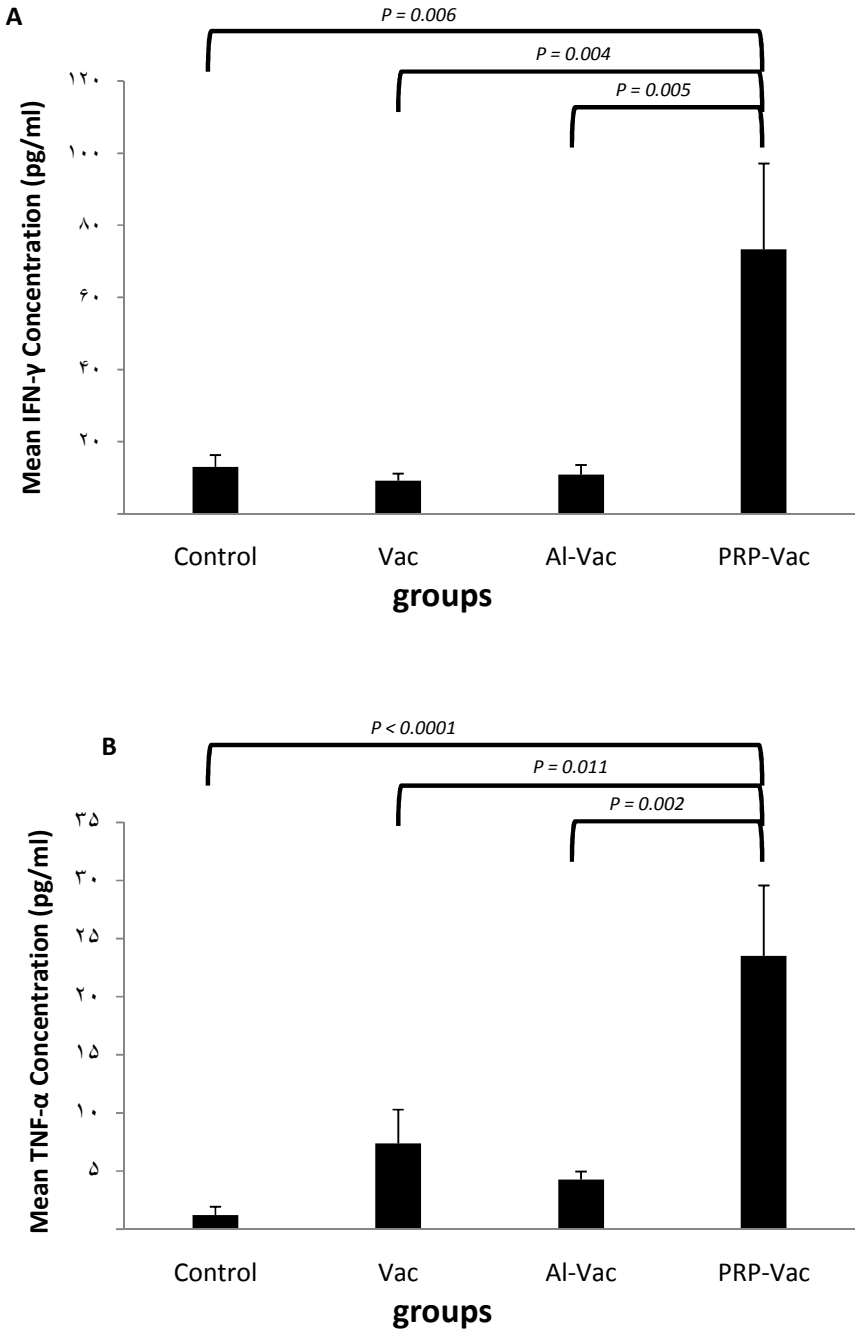

Figure 2. Effect of PRP on cytokine responses.

Two weeks after the last immunization, TNF- α in the sera and IFN-γ in spleen cell supernatants of immunized BALB/c mice were measured by ELISA. IFN-γ (figure 2a) and TNF- α (figure 2b) levels of the mice in PRP-Vac group were significantly higher than those of the mice in Al-Vac, Vac and control groups. The groups are as in figure 1. The values are mean ±SE (n= 5 mice per group).

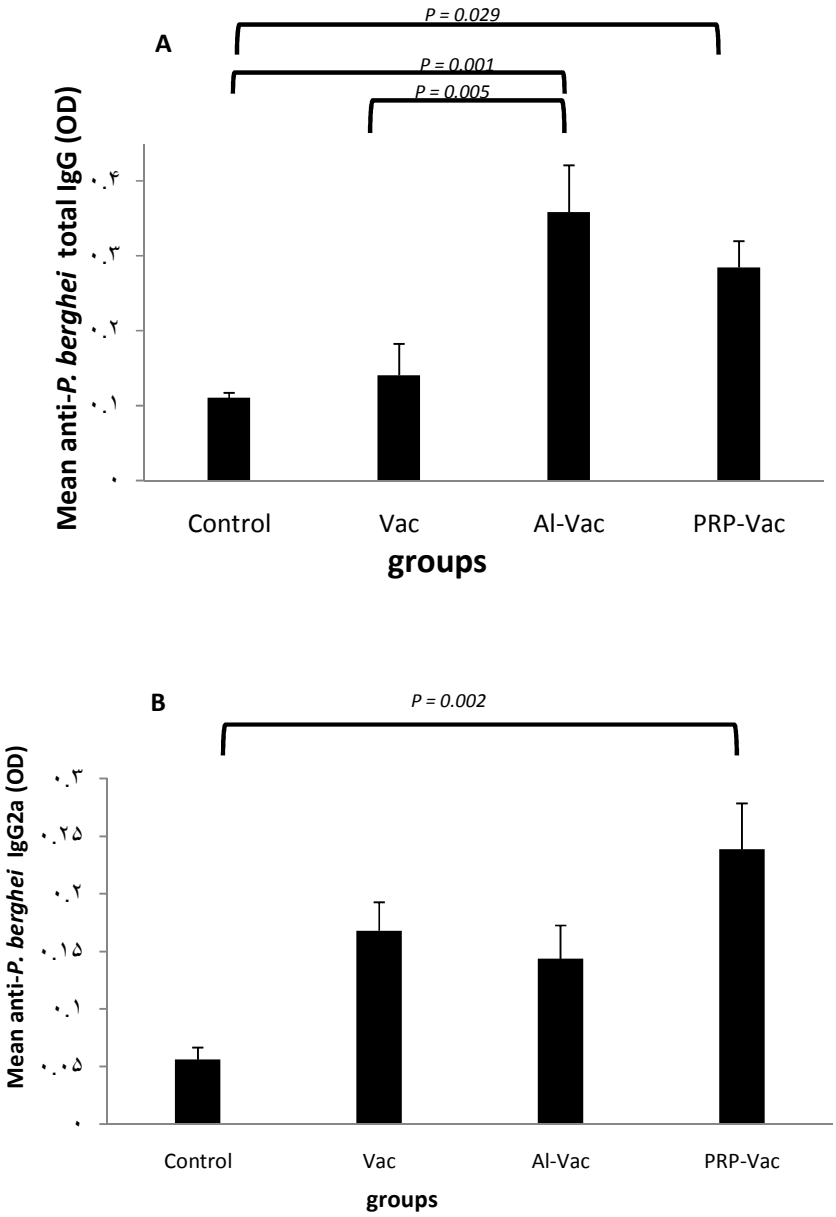

Figure 3. Effect of PRP on production of anti- *P. berghei* IgG.
Two weeks after the last immunization, the serum levels of anti-*P. berghei* IgG2a (figure 3a) and total IgG (figure 3b) antibodies were determined. The groups are as in figure 1. The values are mean ± SE (n= 5 mice per group).

On the ninth day after the parasite challenge, the mean parasitemia of the mice of PRP group was significantly lower than those of the mice in control, Vac and Al-Vac groups . The mean parasitemia of the mice in Al-Vac group was significantly lower than that of the mice in control group and non-significantly less than that of the mice in Vac group.

On both seventh and ninth days after the parasite challenge, the mean parasitemia of the mice in Vac group was less than that of the mice in control group, but the differences were not significant.

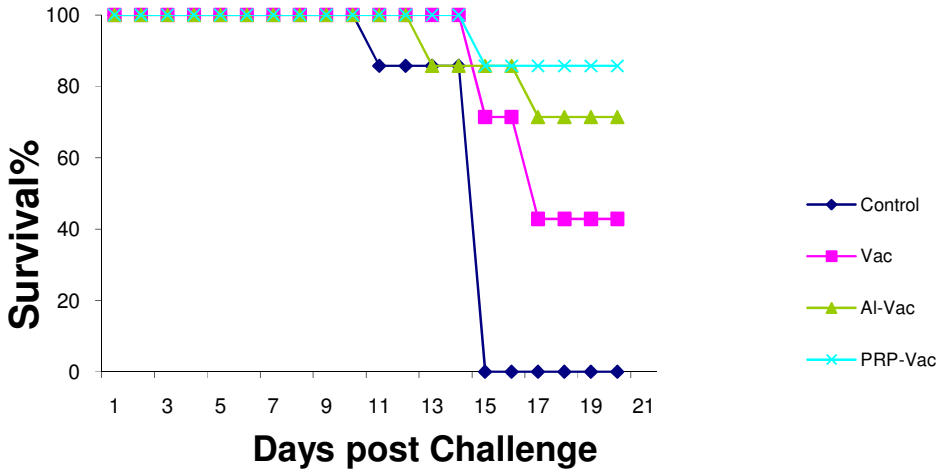

Figure 4. Survival rates of the mice immunized with PBS (control group), PWBSA vaccine alone (Vac group), alum in combination with the PWBSA vaccine (Al-Vac group) and PRP in combination with the PWBSA vaccine (PRP-Vac group). Two weeks after the last immunization, 7 mice from each group were challenged with live *P. berghei* and then their survival rates were recorded daily for 20 days.

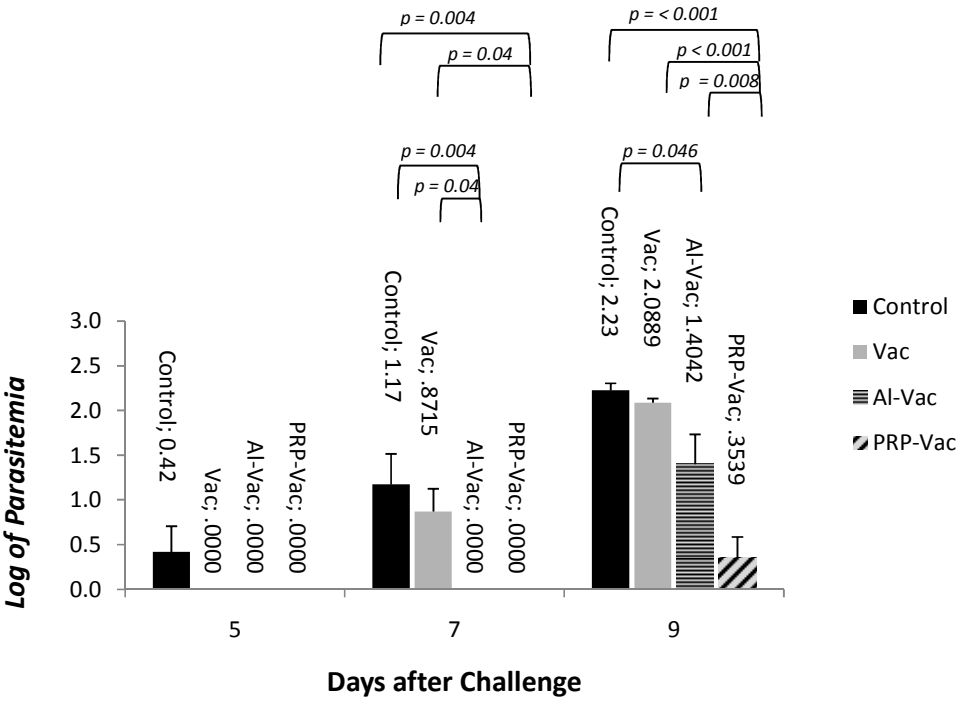

Figure 5. Two weeks after the last immunization, 7 mice from each group were challenged with live *P. berghei* and then parasitemia were determined on the fifth, seventh and ninth days after the parasite challenge. Parasitemia was highly skewed to higher values and was transformed by $\log_{10}$ to normalize the distribution. The groups are as in figure 1. The values are mean ± SE.

DISCUSSION

The aim of vaccination against every pathogen, including plasmodium species, is to stimulate appropriate specific immune responses. Here we investigated the efficacy of administration of PRP in combination with a vaccine model for malaria disease, in inducing protective immune responses.

The results indicated that the administration of PRP, as an adjuvant in combination with the PWBSA vaccine, significantly increased the vaccine-induced production of IFN-γ and TNF-α; lymphocyte proliferation; production of anti-*P. berghei* IgG2a; and improved parasitemia and survival rate against a *P. berghei* challenge. The adjuvant activity of PRP was more than that of alum.

Immunostimulatory effects of PRP observed in the current study are in line with our previous finding showed the adjuvant activity of PRP in combination with *S. typhimurium* vaccine.[24] It also agrees with this finding that treatment of mice with nadolol and ICI118,551, pan β - and β2-blockers, respectively, by osmotic pump during the course of Influenza A virus infection, augment antiviral TCD8 responses.[28] Furthermore, our finding is in line with the other study indicated that administration of long acting PRP and HSP-70 Rich tumor lysate reduced tumor growth and enhanced immune response against fibrosarcoma in BALB/c mice.[29] Binding of β-adrnergic receptor blockers to the β-adrnergic receptors on innate immune cells and lymphocytes could be the cause of the immunostimulatory effects of these agents that were observed in the previous studies.[22] However, regarding the half-life of PRP,[30,31] the adjuvant activity of PRP observed in the current study and also our previous study[24] may only be due to the effects of PRP on the innate immune response, which in turn influences adaptive immunity. Ultimately, PRP would affect the adaptive immunity indirectly, without binding to its receptors on effector T and/or B lymphocytes.

A possible mechanism for PRP adjuvant activity is that PRP blocks β2-adrenergic receptors and this blocking would accelerate local inflammation via inhibiting the effects of APCs-derived catecholamines or catecholamines from other sources such as sympathetic nerves or adrenal glands.[17-21]

Inhibition of regulatory T lymphocytes (Tregs) is one of the other possible mechanisms for adjuvant activity of PRP.[32-34] It has been suggested that dendritic cell (DC) maturation and the expression of costimulatory molecules can be inhibited by Tregs. This in turn decreases ability of DCs to activate effector T cells.[35-37] So it is possible that vaccine-induced immune responses may be enhanced by PRP-induced inhibition of the interaction of Tregs with DCs. This suggested mechanism agrees with findings indicating that inhibition Tregs is the mechanism by which administration of CCR4 antagonist as adjuvant accelerates vaccine-induced immunity.[38,39]

Administration of PRP as an adjuvant may activate APCs via the above-mentioned mechanisms. This would result in the presentation of PWBSA by activated APCs. However, because of the half-life of PRP,[30,31] there is probably little or no PRP in the environment when the APCs present PWBSA. Therefore, it is possible that previously activated APCs induce the Th1 deviated and pro-inflammatory immune response.

The finding of the current study and our previous study about adjuvant activity of PRP and also our previous findings about adjuvant activities of naloxone[40-44] emphasize that at the time of uptaking and processing of an antigen by APCs, the local microenvironment has a crucial role in the orientation of subsequent specific immune response against the antigen.[45,46]

Our results indicated that administration of the PWBSA vaccine alone or in combination with alum failed to induce significant production of IFN-γ. These findings were expectable because alum is a poor inducer of Th1 responses.[8]

In conclusion, PRP as an adjuvant in PWBSA vaccine can shift the immune response to Th1 and enhance cellular immunity. As PRP is approved for human use,[47] it may be considered as a new and relatively safe adjuvant for eliciting effective vaccine-induced Th1 immune responses to malaria. To our knowledge, this study is the first one in the literature to evaluate the adjuvant activity of a β-adrenergic antagonist for use in combination with an anti-malaria vaccine. Therefore, follow-up studies are needed to confirm these results and to examine adjuvant activity of PRP when combined with vaccines against other microbes. Furthermore, as both cellular and humoral immune responses are important in anti-malaria immunity,[16] it is highly possible that using mixture of alum and PRP as adjuvant in combination with an anti-malaria vaccine elicits a stronger immunity comparing

with that of PRP alone, because we previously showed administration of the mixture of alum and PRP as an adjuvant in combination with heat killed *Salmonella typhimurium* elicited the vaccine-induced cellular and humoral immune responses.[24]

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