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Research Note

A recombinant 19 kDa *Plasmodium berghei* merozoite surface protein 1 formulated with alum induces protective immune response in mice

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Abstract. We investigated the immunogenicity of recombinant rMSP1 (rPbMSP1) that was generated from *Plasmodium berghei*. The rPbMSP1 formulated in alum was found to be immunogenic which induced high levels of specific anti-rPbMSP1 antibody. The IgG2a response predominated over IgG1 during the challenge infection in the vaccinated mice. Mice vaccinated with rPbMSP1 in alum mounted significant protective immunity against challenge infection ($P<0.01$). On day 121 after the booster, three out of ten mice immunized with rPbMSP1 in PBS survived parasite infection ($P < 0.05$) and eight out of ten mice vaccinated with r MSP1 in alum did ($P < 0.01$). Hence, immunization with MSP1 in alum obviously has conferred protective effects, which prevented death from *P. berghei* lethal infection in mice ($P < 0.01$). These observations provide an excellent model for clinical assessment of this formulation in human subjects.

Due to wide spread and increasing resistance of parasites to anti-malaria drugs and the resistance of *Anopheles* mosquito vectors to insecticides, malaria poses a serious health problem in tropical countries (WHO, 1999). It is estimated that between 300 to 500 million clinical cases and 2.7 million deaths per year are due to malaria. A number of protective antigens have been identified in animal models utilizing several kinds of adjuvants, most of which are toxic and cannot be applied in humans. Further more, the purified antigens tend to lose immunogenicity. Therefore, an effective and safe adjuvant for human use is one of the keys for successful malaria vaccines.

Antigen of the invasive blood stage of the parasite, the merozoite has been proposed as a vaccine target (Howard & Pasloske, 1993; Diggs *et al*., 1993). This molecule has been identified in almost all of the *Plasmodium* sp. that infect humans, simians and rodents (Holder *et al*., 1981; Hall *et al*., 1984). The major constituents of the merozoite surface are polypeptides derived from a high molecular mass precursor protein, merozoite surface protein 1, MSP1. This protein is synthesized during schizogony and its products are localized on the surface of extracellular merozoites. Post-translational proteolytic processing of the precursor molecule generates fragments but only the

19 kDa fragment is retained on the surface of merozoites throughout invasion of the erythrocytes. All other fragments being shed before or at this event. This 19 kDa fragment is the target of invasion inhibiting antibodies (Blackman *et al.*, 1990).

Recently we have cloned a gene from *Plasmodium berghei* which express the 19 kDa MSP1 protein. The recombinant protein from this gene was produced in *Escherichia coli* and it is designated as rPbMSP1. Purification of rPbMSP1 from the supernatant was performed according to the procedure described by Weiqing *et al.* (2004) which consists of three steps, *viz* phenyl hydrophobic interaction, ion-exchange (DEAE) and gel filtration (Superdex 75; Pharmacia Biotech, Piscataway, NJ) chromatography. Briefly, the fermentation supernatant filtered through a 2.0 um filter was mixed with $(NH_4)_2SO_4$ to a final concentration of 0.8M and adjusted to pH 7.2. The material was applied to a 1.5L phenyl hydrophobic interaction column. The resin was equilibrated in the buffer A (10mM PB, 0.8 M pH 7.2 (NH4)SO4). The protein was eluted to 10 mM PB. The elution was then fractionated by ion exchange chromatography on a 500-ml DAEA column. The protein was eluted with buffer B (10 mM PB, 0.35 M NaCl (pH 7.2). The final step of the purification was performed on a 1.7-L Superdex 75 column and the protein was eluted with buffer C (10mM PB, 0.15 M NaCl pH 7.2). To purify the individual proteins, culture supernatants were dialyzed against buffer D (50 mM NaH_2PO_4, 300mM NaCl (pH 8.0) and then applied directly to Ni^{2+} chelate columns. The resin was extensively washed with 20mM imidazole hydrochloride in buffer D and the proteins were eluted with 250 mM imidazole hydrochloride in buffer D.

BALB/c female mice were used and bred in the Animal Resource Centre, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Experimental mice were 8–15 weeks old at the time of the experiments and they were used in vaccination trials against several challenge infections. The sacrifice of mice was in accordance with guidelines on animal ethics of the Faculty.

Highly virulent *P. berghei* NK65 parasites were used to initiate malaria infection. To prepare parasites for infection, the thawed cryopreserved stabilate of parasites were inoculated into naïve mice serving as sources of parasites. After three days, parasites were harvested from the mice and then intraperitoneally inoculated into experimental mice at a density of either 10^6 or 10^5 parasitized erythrocytes per mouse. Parasitemia in mice were examined daily by microscopic detection of Giemsa-stained blood smears. Levels of parasitaemia were calculated as the percentage of parasitised erythrocytes per 10,000 erythrocytes (parasitaemia percentage). Undetectable parataemia indicated that no parasitised erythrocyte was observed among 10,000 erythrocytes.

Enzyme linked immunosorbant assay (ELISA) were performed to determine the antibody responses generated in mice immunized with rPbMSP1. Antibody titer determination was on the basis of pre-immune sera giving an O.D of 0.05±0.014 at a dilution of 1:200 and an OD cut-off of 0.1 (mean ± 2SD). To detect subclasses of mouse IgG, an ELISA was performed with secondary antibodies (monoclonal antibodies) specific for mouse IgG1, IgG2a, IgG2b, IgG3 and IgG4 conjugated with horseradish peroxidase at dilutions of 1:1000 and sera dilutions used were 1:10,000 for mouse subclass assay. All antibodies were purchased from BD Pharmingen International, Japan and used according to the manufacturer's recommended concentrations. The assay results were evaluated based on OD values determined by a microplate reader (Bio-Rad Laboratories). O.D cut off selected was the same as described above.

Recombinant MSP1 protein from the 19 kDa *P. berghei* was produced in *E. coli* (Angov *et al.*, 2003). Alum was stored under controlled conditions and used within 16 days after formulation. Lyophilized rPbMSP1 was mixed with alum on the day of injection. Each vaccine formulation, containing 10 ug was

administered through IP route.

In the present study, to provide basic data for a DNA vaccine strategy in malaria infection, we evaluated the immunogenicity of rPbMSP1 vaccine formulated with alum adjuvant and the survival of vaccinated mice against challenge infection. Observations during challenge infection of vaccinated mice were compared with those during acute infection of naïve mice. Naïve infected mice usually died of high parasitemia within 7 to 10 days when parasitemia reached between 40% to 60%.

After 30 days, the same amount of each sample was injected through IP to boost immune response. On day 121 after the booster, mice were challenged with 10^4 *P. berghei* parasitized erythrocytes in PBS through the IP route. The course of infection was monitored by microscopic examination of tail blood smears stained with Giemsa. Fisher·s T-test statistically examined the levels of survival among groups of mice immunized.

Several significant findings have emerged from this study. The *E. coli* host strain, origami (DE3) or BL21 Star™ was used to express the recombinant fusion protein (rPBMSP). After 3 hour time point of inducing IPTG (0.1 mM), as expected, an intense protein band with a molecular weight of about 19 kDa (Fig. 1, lane 1) was observed compared to negative controls (lane 2 and 3). This is a highly immunogenic antigen which has been reported to be protective against lethal challenge and inhibit merozoite invasion *in vitro* (Chappel & Holder, 1993).

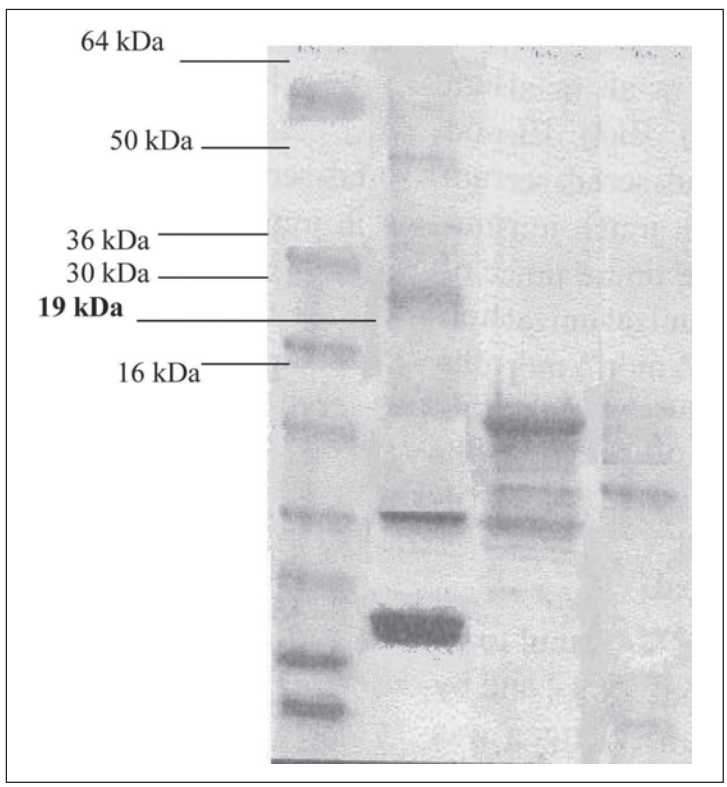

Figure 1. SDS-PAGE analysis of Recombinant *MSP-1* antigen expressed in BL21 Star™ *E. coli*.

Lane 1: Recombinant *MSP-1* antigen (pET TOPO/*MSP-1*).
Lane 2: The vector without insert as negative control.
Lane 3: *E. coli* without vector and insert negative control.

We evaluated the serum IgG responses to infection by comparing IgG values for infected mice and vaccinated mice. The stronger the IgG response, the bigger the difference between test and normal IgG values. In the vaccinated mice, the difference between test and normal IgG2a O.D was greater than the IgG1 O.Ds. This is illustrated in Fig. 3 which shows that the IgG2a O.D. curve is above the IgG1 curve. Figure 2 shows the level of IgG2a is also above that of IgG1 in infected naïve mice. Therefore, the IgG2a response predominated over IgG1 during the challenge infection in the vaccinated mice. There was no elevation of titer with other isotypes. The suppressed parasitemia in the vaccinated mice may have resulted from the enhancement of the effector mechanism of protective immunity due to vaccination with rPbMSP1 vaccine. Regarding to protective immunity in this study, the dominance of IgG2a that may have augmented the Th1 response to *P. berghei* challenge infection in the vaccinated mice. This finding is parallel with reports of Waki *et al.* (1992) and Akanmori *et al.* (1994) on the strong relationship of IgG2a with Th1 cells in protective immunity against *P. berghei*.

After the challenge on day 121 and also after the booster, three out of ten mice ($P < 0.05$) immunized with rPbMSP1 in PBST survived parasite infection (Fig. 4) and nine out of ten mice ($P < 0.01$) vaccinated with r PbMSP in alum did (Fig. 5). Protective effects of rPbMSP in alum immunization obviously has prevented death in eight out of ten mice ($P < 0.01$).

The merozoite surface protein effective induction of immune responses often requires co-administration of adjuvant or immunomodulators (Kumar *et al.*, 2000). The results from this study indicated the potential of MSP-1 recombinant antigens as suitable candidate antigens for anti-malaria vaccine formulation especially if it is formulated in alum. Adjuvant selection for clinical use is an important issue in vaccine development.

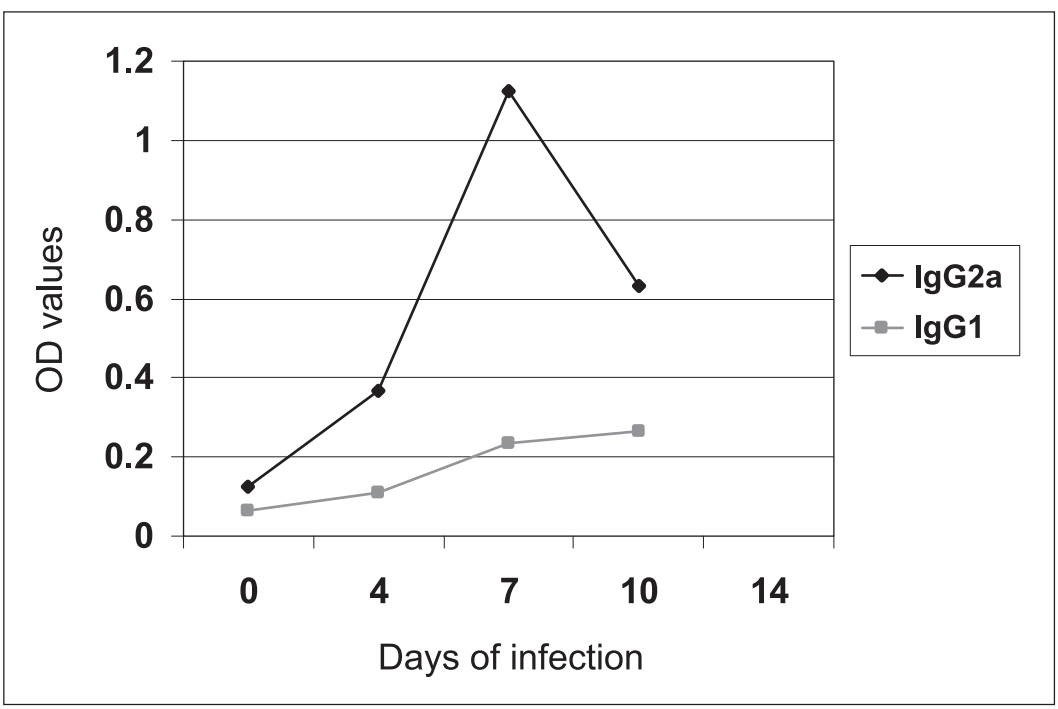

Figure 2. Responses of IgG1 and IgG2a during acute *P. berghei* infection of control mice. The curve for IgG2a is located above that of IgG1. All mice died between 8 to10 days after infection whereby parasitaemia range between 40% to 60%.

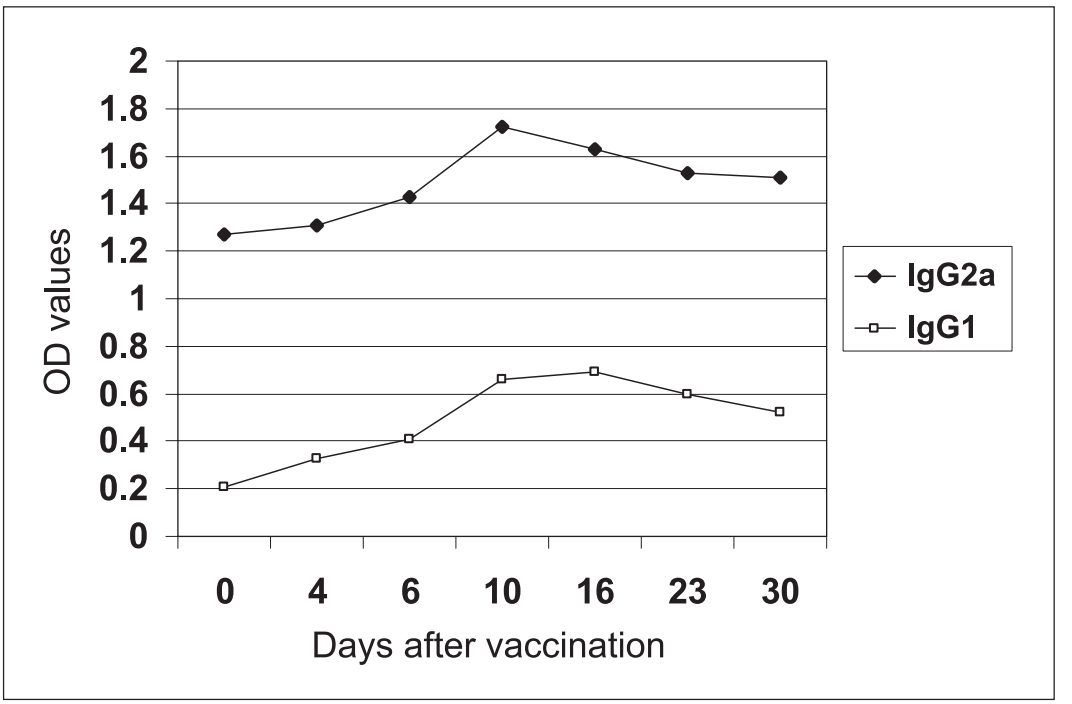

Figure 3. Responses of IgG1 and IgG2a after vaccination with rPbMSP1 plus alum. The curve for IgG2a is located above IgG1.

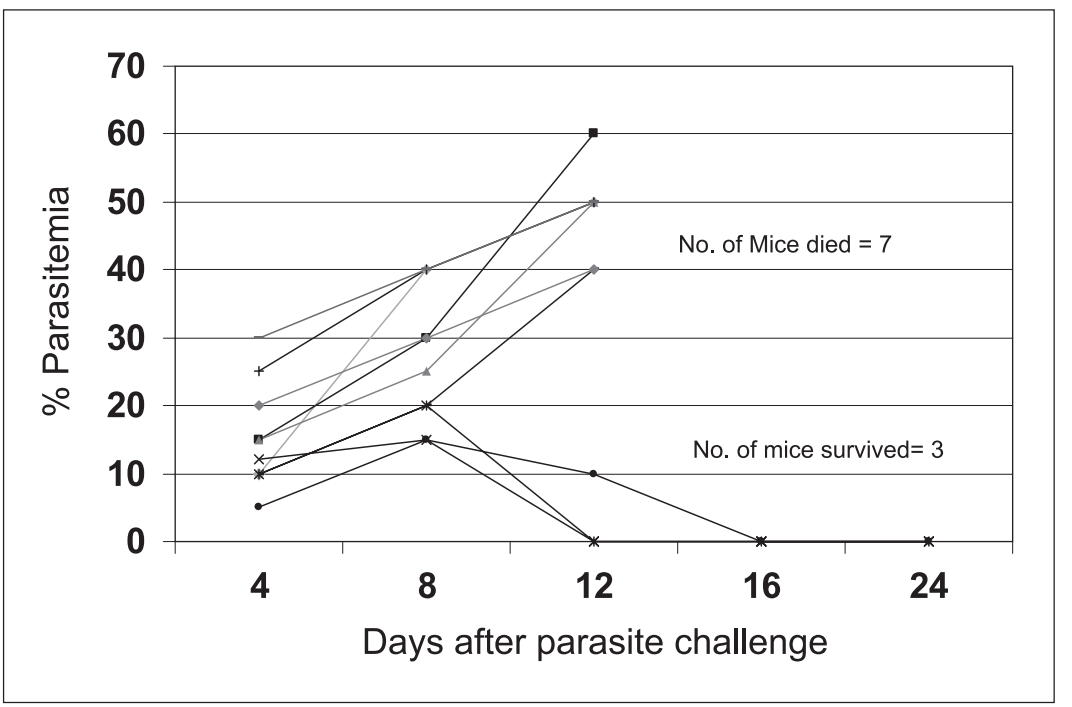

Figure 4. Course of infection of *P. berghei* in female BALB/c mice immunized with rPbMSP1 only. After the challenge on day 121 and after the booster, 3 out of 10 mice survived parasite infection and cleared of parasitemia ($P<0.05$).

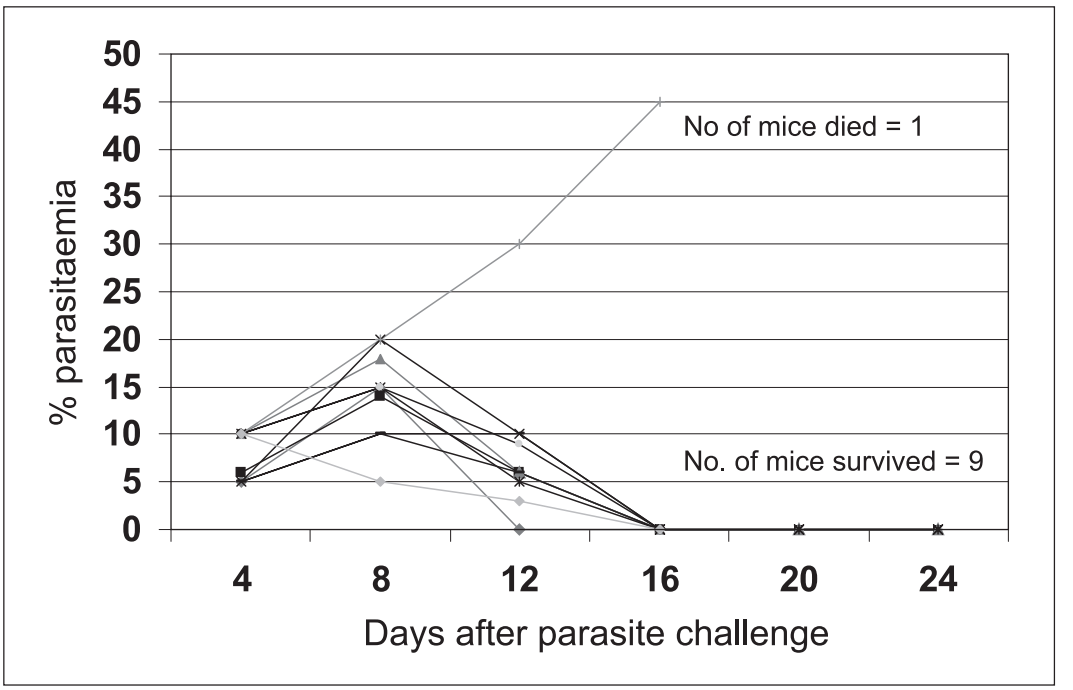

Figure 5. Course of infection of *P. berghei* in female BALB/c mice vaccinated with rPBMSP1 plus alum. After the challenge on day 121 after booster, 9 out of 10 mice vaccinated with rPbMSP1 plus alum survived (P<0.01).

Since its first use in the 1940s, alum is still the only adjuvant approved by the US Food and Drug Administration. Freund's adjuvants are protective in animal models, but not suitable for human use (Egan *et al.*, 2000).

These observations provide an excellent frame work for assessment of other boosters such as interleukins. It is currently possible to obtain recombinant parasite-proteins utilizing the expression system of foreign antigens in *E. coli* (Dally & Long, 1993), baculovirus (Chang *et al.*, 1996), *Saccharomyces cerevisiae* (Hirunpetcharat, 1997) and *Salmonella* (Toebe *et al.*, 1997). The administration of naked plasmid DNA encoding a specific protein antigen has been reported to generate both humoral and cell-mediated immune responses (Wolfe *et al.*, 1990; Sedegath *et. al.*, 1994). DNA vaccine is less toxic because it can be applied without adjuvant (Donnelly *et al.*, 1997). Combination of recombinant proteins with adjuvant elicit more efficient and long-lasting immune responses than rPbMSP1 vaccine alone. Maintainence of high antibody titers is necessary for protection against malaria blood stage parasites.

The administration of naked plasmid DNA encoding a specific protein antigen has been reported to generate both humoral and cell-mediated immune responses. Combination of recombinant proteins with rBCG might elicit more efficient and long-lasting immune responses than rBCG vaccine alone. Experiments on the combinative effects of rPbMSP1 and interleukins and plasmid DNA including the gene encoding MSP1 are under progress in our laboratory. The development of the adjuvant is an important step in developing a successful vaccine. The present study provides reasonable basis for further studies of anti-malarial vaccine development.

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