43 kDa and 66 kDa, two blood stage antigens induce immune response in *Plasmodium berghei* malaria

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The hunt for an effective vaccine against malaria still continues. Several new target antigens as candidates for vaccine design are being explored and tested for their efficacy. In the present study the sera from mice immunized with 24,000 × g fraction of *Plasmodium berghei* has been used to identify highly immunogenic blood stage antigens. The protective antibodies present in immune sera were covalently immobilized on CNBr activated sepharose 4B and used for affinity chromatography purification of antigens present in blood stages of *P. berghei*. Two polypeptides of 66 and 43 kDa molecular weights proved to be highly immunogenic. They exhibited a strong humoral immune response in mice as evident by high titres in ELISA and IFA. Protective immunity by these two antigens was apparent by *in vivo* and *in vitro* studies. These two proteins could further be analysed and used as antigens in malaria vaccine design.

**Keywords:** Antibodies, Antigens, Immune response, Malaria, *Plasmodium berghei*, Vaccine

Malaria is one of the deadliest vector borne disease in the world. It is responsible for afflicting about 250 million people annually and resulting in nearly 1 million deaths. More than six decades have passed in search of an effective vaccine against malaria and there is still no hope. Worldwide understanding of the malaria parasite’s biology and its allied molecular interactions with the host could possibly provide necessary cues for designing of effective antimalarial drugs and vaccines. Along with this it is now clear that understanding immune responses to *Plasmodium* in rodent models is perhaps the key to unlock new vaccine strategies. Further, new antigens have to be explored which will act as target candidates for vaccine design.

Role of antibodies in protective immunity against malaria is well established. The antibodies present in the naturally or experimentally immune individuals can be used to identify antigens. In the present study immunoaffinity chromatography which uses immobilized antibodies as affinity ligands specific to either an antigen or a group of structurally related antigens was used to isolate and characterize *P. berghei* proteins displaying strong reactivity against antibodies of mice immunized with 24,000 × g fraction.

**Materials and Methods**

Malaria parasite and animals—The asexual erythrocytic stages of *Plasmodium berghei* (NK-65) were maintained in albino mice *Mus musculus* (BALB/c) procured from Central Research Institute (CRI), Kasauli. The animals were kept in the animal house of Himachal Pradesh University, Shimla where animal care and experiments were carried according to the guidelines of Institutional Animal Ethics Committee (IAEC/Bio/1-2008). Parasite was maintained by inoculating intraperitoneally 1 × 10⁵ *P. berghei* infected erythrocytes in citrate saline [sodium chloride, 0.85% (w/v); sodium citrate, 3.8% (w/v)] from infected to naïve animals. Parasitaemia was monitored by making Giemsa stained thin blood smears and cell - free parasite was isolated from the blood of *P. berghei* infected mice at about 50% parasitaemia. Cell-free *P. berghei* was suspended in 0.25 M sucrose in 0.01M PBS, pH 7.2 and homogenized at 4°C using Potter-Elvehjem homogenizer (Remi, Bombay).

Isolation of 24,000 g fraction—The 24,000 × g fraction of parasite was collected by the subcellular fractionation of the homogenized parasite. A part of the total parasite homogenate was subjected to centrifugation at 600 × g for 15 min at 4 °C. All the centrifugations were carried in Sigma 3K30 centrifuge. The centrifuge pellet was discarded and the supernate was further centrifuged at 10,000 × g for 25 min. Pellet was isolated and the supernate
again subjected to centrifugation at 24,000 \times g for 35 min. Pellet was separated and labelled as 24,000 \times g fraction while the supernate was discarded. The pellet was suspended in PBS (0.01M, pH 7.2), homogenized and used for immunization of mice. Protein was determined spectrophotometrically using bovine serum albumin (BSA) as standard.

**Immunization of mice and collection of sera**—Mice (10) were immunized with the 24,000 \times g fraction. Each mouse was injected, ip with 100 µg of parasite material and 30 µg of saponin on day 0, 14 and 28. A week after the last immunization dose, the mice were sacrificed to collect the immune sera. The immune sera were pooled and stored at -20 ºC for further use.

**Purification of antigen by immunoadsorption**—Different antigenic proteins from the pellet at 24000 \times g (PIII) were isolated by antibody immobilization method. Serum containing antibodies, mixed with PBS, pH 7.2 was added to an equal amount of CNBr activated Sepharose 4B and left overnight at 4 ºC. Protein of uncoupled antibodies was measured spectrophotometrically \( (A_{280}) \) and remaining active sites were blocked by treating the gel with 1 M ethanolamine. The uncoupled antibodies were removed by washing 4-5 times with low pH wash solution (sodium acetate, 0.1 M, pH 4; sodium chloride, 0.5 M) followed by high pH wash solution (sodium bicarbonate, 0.1 M, pH 8.3 and sodium chloride, 0.5 M). After washings with PBS the antibody immunosorbent was poured onto a glass column. The column was preeluted with elution buffer (0.1 M glycine-HCl, pH 2.6; sodium chloride, 0.15 M) and reequilibrated with PBS, pH 7.2. The antigen solution was then allowed to flow through the column at a speed of 20 mL/h under gravity. The bound antigens were then eluted with the elution buffer (glycine-HCl, 0.1 M, pH 2.6; NaCl, 0.15 M) and collected as 1 mL fractions. Each eluted fraction was immediately neutralized with 45 µL of Tris-HCl 2 M, pH 8.0. All the fractions were stored at -20 ºC till further use. The molecular weights of the purified antigens through immunoadsorption were determined by SDS-PAGE.

**SDS gel electrophoresis**—Parasite proteins isolated by immunoadsorption were resolved by SDS-PAGE using 3% stacking and 10% separating gel. Gels were stained in coomassie brilliant blue.

**Immunization of mice with purified antigens**—The purified proteins eluted after immunoadsorption and the ones which showed similar molecular weights were pooled together. These pooled proteins were used as antigens to immunize mice. Five groups (A, B, C, D and E) of 8 mice each were immunized with pooled antigens. Each mouse was injected 100 µg of purified protein along with 30 µg saponin ip on day 0, 14 and 28 (Table 1). Along with the experimentals, 8 mice were used as placebo controls and injected with 30 µg saponin in PBS, pH 7.2 on day 0, 14 and 28.

**Collection of immune sera and challenge of immunized mice**—Four mice in each group were sacrificed to collect prechallenge immune sera. Sera were stored at -20 ºC till used in various serological assays to determine the level of antimalarial antibodies. A week after the final immunization dose, 4 mice from each group along with placebo controls were injected \( 1 \times 10^5 \) \( P. \) berghei-infected erythrocytes, ip. The course of parasitaemia was monitored daily by preparing Giemsa stained thin blood smears till death of animals or clearance of the parasite from the immunized mice.

**Enzyme linked immunosorbent assay (ELISA)**—Humoral immune response induced in mice to different antigens was analysed by enzyme linked immunosorbent assay (ELISA) for pre- and postchallenge sera.

**Indirect flourescent antibody test (IFA)**—Prechallenge immune sera were also tested by indirect fluorescent antibody test (IFA).

**Immunoblotting**—The total parasite homogenate was resolved by SDS-PAGE using 3% stacking and 10% separating gel. The separated proteins were blotted onto nitrocellulose membrane (NCM) and incubated with prechallenge sera of mice immunized with fractions A, B, C, D and E, 1 h at 37 ºC followed by incubation with goat anti-mouse IgG peroxidase conjugated sera (Genei, Bangalore) as secondary antibody. Antigen–antibody reactions were detected using 0.05% (w/v) diamino benzidine tetrahydrochloride (DAB) in 0.01 M PBS, pH 7.2 and \( \text{H}_2\text{O}_2 \).

**In vitro merozoite invasion inhibition assay**—The short term in vitro culture of \( P. \) berghei was carried out in 24-well culture plates. The percent inhibition of merozoite invasion was calculated as:

\[
\text{Inhibition(\%)} = \frac{\text{Number of rings in experimental culture} - \text{Number of rings in control culture}}{\text{Number of rings in control culture}} \times 100
\]
Results

Purification of parasite antigens and immunization of mice—Prechallenge immune serum from mice immunized with 24000 × g fraction (PIII) was coupled with CNBr activated Sepharose 4B. 52% coupling was obtained after mixing the antibody with CNBr activated Sepharose on ice. 1 mL antigen (1.9 mg/mL) was loaded on a glass column containing immunosorbent. 25 fractions of 1 mL each were eluted with PBS pH 7.2 until no protein was detected in the eluate. The column was then washed with Borate-Saline-Tween followed by washing with PBS, pH 7.2. Bound antigens were eluted with glycine-HCl buffer, pH 2.6 %. 50 fractions each of 1 mL were collected and neutralized with 45µL of 2 M tris-HCl, pH 8.0. The elution profile of purified antigens is given in Fig. 1. The molecular weights of the purified antigens were determined by SDS-PAGE. Fractions 1-8 contained proteins of molecular weights 35, 32 and 29 kDa, fractions 9-14, 23-32 and 43-48 contained 66 and 43 kDa proteins, fractions 19-22 contained 63 kDa proteins, fractions 33-36 contained 43 kDa protein, fractions 37-42 contained 147, 112, 105 and 65 proteins. Antigens/proteins showing similar molecular weights in different fractions were pooled together. 5 groups of 8 mice each were immunized accordingly as shown in Table 1.

Serological analysis—Serological analysis by ELISA showed induced humoral immune response by the pooled fraction B and D containing proteins of molecular weight 66 and 43 kDa respectively. The antibody titres for the pooled prechallenge immune sera of B and D groups were 1:2048 and 1:4096 respectively whereas very low titres were recorded for A, C and E groups (1:128, 1:256 and 1:256 respectively, Fig. 2). Serum from placebo control showed no reaction. The analysis by IFA was specific showing antiparasite antibodies in the prechallenge sera of group B and D (Fig. 3) whereas no reactions seen for groups A, C, E and placebo control.

Immunoblotting—The prechallenge sera from different immunized groups was further analysed by immunoblotting. No bands were recognized by the pooled prechallenge sera of groups A, C and E. Group B sera recognized two proteins of 66 and 43 kDa whereas group D recognized single protein of 43 kDa out of the many proteins in PAGE of the total parasite homogenate (Fig. 4).

In vitro merozoite invasion inhibition assay—The in vitro invasion inhibition assay was performed using prechallenge immune sera of mice immunized with different fractions. The smears prepared with cultures supplemented with various prechallenge immune sera after 21 h of incubation showed that the number of trophozoites and schizonts decreased as compared to 0 hour. After 21 h of culture, no invasion inhibition was observed for pooled sera of Group A mice (Table 2). Whereas 62.5% invasion inhibition was

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<th>Groups/ Fractions</th>
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<th>Molecular Wt.</th>
<th>Protein (µg/mL)</th>
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<tr>
<td>A</td>
<td>1-8</td>
<td>32, 35, 29</td>
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<tr>
<td>B</td>
<td>9-14, 23-32, 43-48</td>
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<td>E</td>
<td>37-42</td>
<td>147, 112, 105, 65</td>
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![Fig. 1—Elution profile of purified antigens eluted with glycine-HCl buffer.](image1)

![Fig. 2—Level of antiparasite antibodies of mice immunized with A, B, C, D and E fraction, prechallenge immune sera collected on day 35 and post challenge immune sera collected on day 60 post immunization.](image2)
observed with sera of Group B. Sera of group C and E did not show any invasion inhibition. However, sera of group D showed 75% invasion inhibition (Fig. 5).

Challenge of mice—Four mice in each group were challenged with $1 \times 10^5$ *P. berghei* infected erythrocytes. Course of parasitaemia in these mice is depicted in Fig. 6. All the mice of groups A, C, E and placebo control died because of infection. However, in group B three mice survived after challenge and the parasitaemia remained low (max = 4.08%). In group D again three mice survived and maintained a low parasitaemia (max = 2.27%). Postchallenge immune sera of mice which survived challenge were analysed for the antimalarial antibodies by ELISA. Group B and D showed a high titre of 1:8192.

**Discussion**

At present there is a global understanding of the malaria parasite’s biology and its associated molecular interactions. The existence of naturally acquired immunity and findings that blood stage immunity can be developed by exposure to repeated infections present a strong basis for the identification of those antigen targets which elicit high levels of antibody responses. Therefore, new methodologies...
need to be developed to isolate blood stage antigens by using parasite-targeted antibodies found in immunized individuals.

To achieve this immunoaffinity chromatography technique was used in the present study. Mice were immunized with 24,000 × g fraction of *P. berghei* which has already proven highly immunogenic. Sera of these immunized mice were collected and used for the purification and characterization of parasite proteins. Purified fractions were collected and proteins having similar molecular weights were pooled together and used for *in vivo* immunization of mice.

Fractions B and D containing two proteins of 66 and 43 kDa molecular weights proved to be highly immunogenic. They exhibited a strong humoral immune response in mice. Protective immunity was evident as out of 8 mice in these two groups, six survived after challenge with live parasite infection. Humoral immunity was evident from the high antibody titres in the pre and post-challenge sera of groups B (66 and 43 kDa) and D (43 kDa) as determined by ELISA and IFA. The other groups A, C and E showed very low antibody titres in ELISA and no reactions in IFA. Immunoblotting further confirmed that the antibodies present in immunized individuals were directed against 66 and 43 kDa proteins.

Several studies on various other species like *P. yoelli*, *P. knowlesi* and *P. falciparum* have reported partial or complete protection against experimental malaria by immunization with antigens of molecular weights 66 and 43 kDa. Along with this the plasmepsin or aspartic protease of *P. berghei* also has a molecular weight of 43 kDa. Plasmepsins have antigenic properties and antibodies are elicited against plasmepsins.

*Plasmodium* blood stage life cycle progresses by invasion of RBCs, rupturing of infected RBCs and by again reinvasion of healthy RBCs by merozoites. Invasion by merozoite depends on merozoite surface protein (MSP1). Along with this it also involves additional distinct receptor–ligand interactions. Keeping this in mind an important strategy to curb blood stage malaria is to immunize with such antigens that elicit immune responses targeting the extracellular merozoites and further preventing the invasion of erythrocytes. *In vitro* invasion indicated inhibition of the red blood cells by the pre-challenge sera of Group B and D. 62.5% invasion inhibition was observed with sera of Group B whereas sera of group D showed 75% invasion inhibition. Sera of groups A, C and E did not show any invasion inhibition. Merozoite invasion of red cells *in vitro* by immune sera has been shown in various earlier studies.

The limited success of generating protective immunity against malaria using subunit vaccines composed of one or a few parasite proteins (or protein fragments or domains) has prompted the search for new antigenic proteins able to induce protective immunity. The circulating antibodies against blood stage malaria in experimentally immunized individuals constitute a major component of the...
immune response. This was the rationale behind the idea of using immunized mice as a source of antibodies to search out *P. berghei* antigens present during blood stage infection. Further identification and characterization of these antigens should be carried out as this study is just a step towards the larger goal. As the protective immune responses in malaria have many elusive features, the present results, in a limited way, provide a new direction for studies designed to identify new antigens in human malaria parasites as vaccine targets and reveal that a variety of proteins may offer efficient B cell-based protective immunity. Hopefully, a vaccine based on such a variety of antigenic proteins elicited during the parasite blood stage could be the key to effective protection against malaria.

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**References**


