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Serodiagnosis of Immune Response Exhibited by 24,000 g Fraction in Rodent Malaria

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ABSTRACT

Immunity to malaria can be induced by experimental infection in rodents. A large number of malaria antigens have been identified having their role in protective immune responses to malaria. In this study, mice immunized with soluble antigens of 24,000 g sedimented fraction exhibited strong humoral immune response and this was evaluated by serodiagnostic assays. When 24,000 g fraction were subjected to SDS-PAGE, showed protein bands with molecular weights ranging from 24-80 kDa. It appears that some of these proteins act as target antigens. The immune sera obtained from immunized mice were subjected to ELISA, IFA and *in vitro* invasion inhibition assay. The antibody titres ranged from 1:2048-1:16384 in immune sera.

Key words: 24,000 g fraction, apicomplexan parasite, immunization, immune response

INTRODUCTION

Malaria caused by the Apicomplexan parasite, *Plasmodium*, remains a serious public health threat in tropical and sub-tropical countries. Strategies like vector-control, chemotherapy and development of an effective malaria vaccine have been used to control and eradicate malaria. However, the increasing spread of drug resistance in *Plasmodium falciparum* and insecticide resistance in *Anopheles* resulted in the resurgence of this dreaded disease (WHO, 2012). Effective control of this disease is possible by better understanding of basic biology of the parasite (Hakimi *et al*., 2013).

Immunity to malaria is species and stage specific and both humoral and cell mediated responses are activated during infection (Dodoo *et al*., 2011). Anti-*P. falciparum* IgG antibodies play important role in the elimination of asexual blood stages of parasite. *In vitro* study showed that human IgGs recognize either infected erythrocytes or merozoites which act in cooperation with monocytes to eliminate the parasite (Afridi *et al*., 2012). A large number of parasite molecules have been identified and many of them are at advanced stage of malaria vaccine strategies but still new antigens need to be explored for an effective malaria vaccine. In this study, the immunogenicity and protective efficacy of 24,000 g fraction of *Plasmodium berghei* was evaluated.

MATERIALS AND METHODS

Maintenance of parasite: *Plasmodium berghei* was maintained in white Swiss mice, *Mus musculus* (BALB/c) as per guidelines of Institutional Animal Ethics Committee of Himachal Pradesh University, Shimla. The asexual erythrocytic stage was maintained by

inoculation of 1×10^5 *P. berghei*-infected erythrocytes from the infected individual to the naive (Banyal *et al.*, 1991). Parasitaemia or percent infection was monitored by blood smear examinations.

Isolation of cell-free parasite and preparation of cell free parasite homogenate: Cell-free *P. berghei* was isolated according to the method of Banyal and Fitch (1982) using 0.2% (w/v) saponin in 0.01M PBS, pH 7.2 (Kapoor and Banyal, 2011).

Differential centrifugation: Differential centrifugation of the homogenized parasite was carried out by the method of Banyal *et al.* (1979). Total parasite homogenate (HOM) was prepared by suspending the cell free parasite in sucrose (0.25 M in PBS, pH 7.2) and homogenized. Homogenate was centrifuged at 600 g for 15 min (Sigma 3 k 30 centrifuge). The sediment was discarded and the supernate was further centrifuged at 10,000 g for 25 min. Again the sediment was discarded and the supernate was centrifuged at 24,000 g for 35 min. The resulting sediment was separated and used as 24,000 g fraction. All centrifugations were carried out at 4°C in a refrigerated centrifuge.

Protein estimation: Protein was estimated spectrophotometrically using Bovine Serum Albumin (BSA) as standard (Banyal and Kumar, 1994).

Immunization of mice: Five mice were immunized with total parasite homogenate and another group of five mice immunized with 24,000 g fraction. Each mouse was given 100 μg of protein along with 30 μg saponin as adjuvant in 0.01 M PBS, pH 7.2 intraperitoneally. This was followed by two booster doses on day 14 and day 28. Placebo control group of three mice was injected only 30 μg of saponin in 0.01 M PBS, pH 7.2. Nine days after last immunization dose, mice from each group were sacrificed to collect immune sera. Sera were stored at-20°C for further use.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Total parasite homogenate and 24,000 g fraction were subjected to SDS-PAGE according to the method of Sharma and Banyal (2011) using 3% stacking and 10% separating gels. Molecular weights of the proteins were calculated by the Gel Documentation System.

Enzyme linked immunosorbent assay (ELISA): ELISA was carried out in 96-well polystyrene microtitre plates according to the method of Banyal and Inselburg (1985).

Indirect fluorescent antibody test (IFA): IFA was performed according to Upma and Banyal (1998) using Fluorescein Isothiocyanate (FITC, Genei).

***In vitro* invasion inhibition assay:** The short-term *in vitro* culture of *P. berghei* was carried out as given by Upma and Banyal (1998) using RPMI-1640 (Gibco) as culture medium.

Culture medium-RPMI-1640 supplemented with 0.06% HEPES (N-2-hydroxyethyl piperazine-N' 2-ethane sulphonic acid), 5% (w/v) sodium bicarbonate, antibiotics-gentamycin (50 μg mL^{-1}), penicillin (100 μL mL^{-1}) and streptomycin (100 μg mL^{-1}) was used as culture medium. The pH of incomplete medium was adjusted to 7.4 and filtered through 0.22 μ millipore syringe filter under sterile conditions, 10% (v/v) inactivated Foetal Calf Serum (FCS) was added to incomplete medium and the complete medium was prepared.

Invasion inhibition assay: The culture was carried in 12 well culture trays. To each well 1 mL of complete medium having 3% haematocrit and 0.3-0.8% parasitaemia along with 50 µL of normal/immune mice serum was added. The trays were then shaken gently to mix the contents.

A little of sample was taken, centrifuged and from resulting pellet portion 0 h smears were prepared. The culture trays were placed in a candle jar at 37°C in an incubator. After 21 h of incubation, the trays were removed from incubator and smears from each well were prepared after centrifugation of different samples. Smears were fixed in methanol and stained with Giemsa stain. A differential count of the parasite (ring, trophozoites and schizonts) in smears was done. The percent inhibition of merozoite invasion was calculated as:

$$\text{Inhibition (\%)} = 100 - \frac{\text{No. of rings in experimental culture}}{\text{No. of rings in control culture}} \times 100$$

RESULTS

Plasmodium berghei subjected to differential centrifugation and 24,000 g fraction analysed by SDS-PAGE revealed many proteins of molecular weights ranging from 24-84 kDa (Fig. 1). Sera obtained from mice immunized with 24,000 g fraction analysed by ELISA gave anti malarial antibody titre of 1:16384, 1:16384, 1:8192, 1:4096 and 1:4096 in mouse number 1, 2, 3, 4 and 5, respectively. The immune sera of mice immunized with total parasite homogenate showed ELISA titre of 1:4096 in mouse number 2, 4 and 5 while the remaining mouse number 1 and 3 showed ELISA titre 1:8192 (Table 1). Immune sera of mice immunized with 24,000 g fraction gave strong immunofluorescence reaction observed under UV light. While the reference negative or normal serum did not show any fluorescence (Fig. 2).

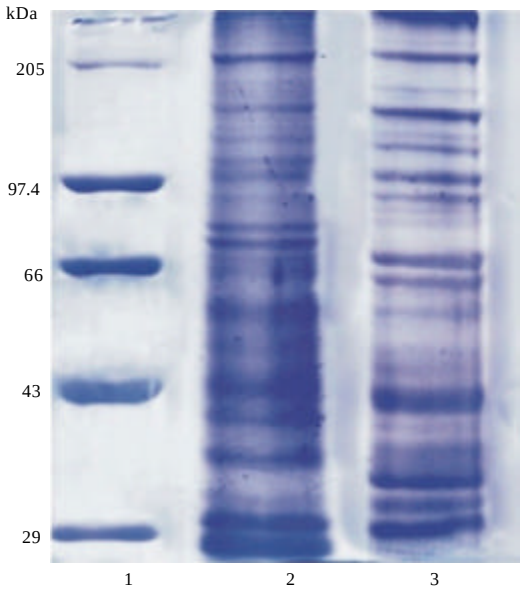

Fig. 1: SDS-PAGE of various fractions of *P. berghei* stained with Coomassie brilliant blue. Lane 1 = Protein standard marker, Lane 2 = Total parasite homogenate (HOM) and Lane 3 = Sediment of 24,000 g fraction

Table 1: Table showing levels of antiparasite antibodies in sera of five mice immunized with 24,000 g sediment and in HOM (total parasite homogenate) group

Mouse No.	ELISA titre of mice immunized with 24,000 g fraction	ELISA titre of mice immunized with HOM (total parasite homogenate)
M1	1:16384	1:8192
M2	1:16384	1:4096
M3	1:8192	1:8192
M4	1:4096	1:4096
M5	1:4096	1:4096

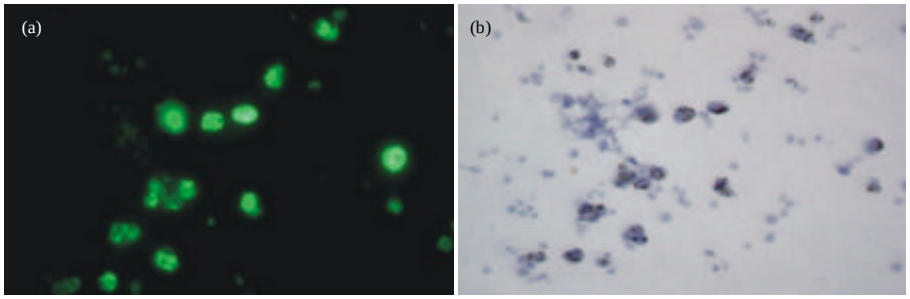

Fig. 2(a-b): IFA test using sera of immunized mice as seen under (a) UV light and (b) Phase contrast (×1000)

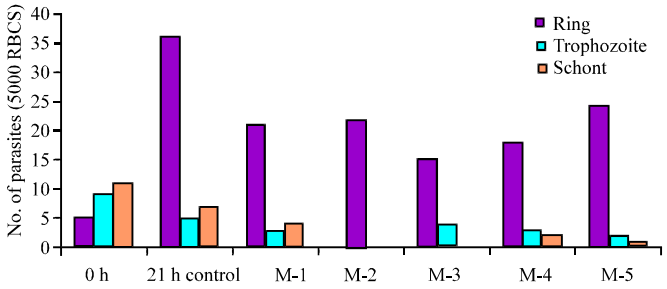

Fig. 3: Histogram showing percent infection in control and sera of five mice (M1, M2, M3, M4 and M5)

The immune sera were also analysed for their anti malarial property by short term *in vitro* invasion inhibition assay. The immune sera obtained from mice immunized with 24,000 g fraction showed significant inhibition of formation of new rings in the culture. Even mouse number 3 and 4 showed more than 50 per inhibition as seen in control culture. The newly formed rings after 21 h incubation were about seven fold more compared to initial culture (Fig. 3).

DISCUSSION

There are certain target antigens which are under investigation for developing effective malaria vaccine. Animal studies have shown the role of various antigens like AMA-1 to stimulate antibody responses which are correlated with reduction of parasite density, effective for vaccine development (Ouattara *et al*., 2010). *Plasmodium* parasites have three sets of secretory organelles at the apical

end-rhoptries, micronemes and dense granules. And the apical proteins associated with these are leading vaccine candidates (Kats *et al*., 2006). Rhoptries and lysosomes are acidic and contains acid-dependent proteases. Lysosomes play role in degradation, recycling and undergo regulated secretion by fusing with the plasma membrane of the cell, releasing their contents into the extracellular environment (Blott and Griffiths, 2002). Host immune responses to malaria parasites is crucial for the effective implementation of new vaccines and drugs (Engwerda and Good, 2005).

In the present study serodiagnosis of immune response in rodent malaria has been undertaken. The mice immunized with soluble antigens of *P. berghei* exhibited strong humoral immune response. Sedimented fraction at 24,000 g contains organelles having hydrolytic enzymes (Banyal *et al*., 1979). Earlier studies (Upma and Banyal, 1998; Pirta and Banyal, 2012) were shown that 24,000 g fraction immunized mice were protected against infection. The 24,000 g fraction when subjected to SDS-PAGE, showed protein bands with molecular weights ranges from 24-80 kDa. It appears that some of these proteins act as target antigens. The immune sera obtained from immunized mice were subjected to ELISA, IFA and an *in vitro* invasion inhibition assay. These assays show the inhibition of antibodies to parasite. All the mice immunized with total parasite antigen, 24,000 g fraction and saponin exhibited high titre of antibodies, as determined by ELISA. ELISA test evaluate the presence of antigen or the presence of antibody in a sample and determine serum antibody concentration. The antibody titres range from 1:4096-1:16384 in sera immunized with 24,000 g fraction. And the ELISA titre for total parasite antigen ranges from 1:4096-1:8192. This shows that the antigen present during inoculation activated the immune system of mice and triggered humoral immune response. The specificity of antibody produced by 24,000 g fraction was also analysed by Indirect Fluorescent (IFA) test. The antigen-antibody reaction was specific as seen through UV and phase microscopy.

Thus 24,000 g fraction induces strong humoral response and the mice immunized with this fraction also showed *in vitro* and *in vivo* protection against malaria infection. This shows that 24,000 g fraction has the important constituent of the parasite, responsible for protection against malaria. This antigen necessitated to be investigated further so as to know its antimalarial role against malaria.

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