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Fractions Isolated by Differential Centrifugation of *Plasmodium berghei* Induce Humoral Immune Response

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ABSTRACT

Plasmodium berghei when subjected to differential centrifugation resulted in various subcellular fractions. Immune sera obtained from mice immunized with PIII fraction (pellet at 24,000 g) showed very high antibody titres detected by ELISA and IFA as compared to other fractions. Mice immunized with these fractions along with placebo controls were also challenged with 1×10^5 *P. berghei* infected erythrocytes. Mice immunized with PIII fraction showed complete *in vivo* protection against parasite. Purification of antigens through immunoadsorption revealed antigens exhibiting protection *in vivo* against parasite challenge. Such antigens may act as useful tool in a fight against malaria.

Key words: *Plasmodium berghei*, malaria parasite, immunization, subcellular fractionation

INTRODUCTION

Malaria is a devastating and quite often deadly parasitic disease afflicting about 250 million people with mortality rate of 1 million deaths annually. It has emerged as one of the top three killers among the vector borne diseases in the world and in addition to its health toll, malaria puts a heavy economic burden on countries where it is endemic (Ayeni, 2011; Amiruddin *et al*., 2012; Mia *et al*., 2012). Reduction in morbidity and mortality as well as possible eradication of the disease will depend to a large extent on safe and effective malaria vaccine.

Groundbreaking advances have been made in understanding the biology of immune mechanisms against *Plasmodium*. Novel features of *Plasmodium* life cycle have been unravelled and immune mechanisms which take place during both infection and immunization, have been explored thoroughly (Douradinha and Doolan, 2011). Most of the vaccines on which the trials are going on belong to one of three categories-attenuated parasite, killed parasite or protein subunits (Banyal and Elangbam, 2006).

Subcellular organelles or cytosolic constituents of malaria parasite posess enzymatic activities (Ibrahim *et al*., 2011) and some of them also elicit immune response activation against the parasite (Sharma and Banyal, 2009; Muller *et al*., 2010; Kapoor and Banyal, 2011a). In the present study the role of different subcellular fractions in inducing humoral immune response in mice against *Plasmodium berghei* has been evaluated and the organelles isolated at 24,000 g provided significant protection in rodent malaria.

MATERIALS AND METHODS

Parasite: Maintenance of asexual erythrocytic stages of *Plasmodium berghei* (NK-65) in BALB/c mice, course of parasitaemia and isolation of cell-free parasite from the blood of *P. berghei* infected mice (about 50% parasitaemia) were carried according to Kapoor and Banyal (2011b). Animal care and experiments were carried according to the guidelines of Institutional Animal Ethics Committee (IAEC/Bio/1-2008). Cell-free *P. berghei* was suspended in 0.25 M sucrose in 0.01 M PBS, pH 7.2 and homogenized at 4°C using Potter-Elvehjem homogenizer (Remi, Bombay).

Subcellular fractionation: Subcellular fractionation of the homogenized parasite was carried out by the method of Banyal *et al.* (1979). A part of the total parasite homogenate was designated as homogenate (HOM) while the remaining parasite material was subjected to centrifugation at 600 g for 15 min at 4°C. All the centrifugations were carried in Sigma 3K 30 centrifuge. The centrifuge pellet (P-I) was discarded and the supernate was further centrifuged at 10,000 g for 25 min. Pellet (P-II) was isolated and the supernate again subjected to centrifugation at 24,000 g for 35 min. Pellet (P-III) was separated while the supernate fraction was designated, S-III. The pellets were suspended in Phosphate Buffer Saline (PBS) (0.01 M, pH 7.2), homogenized and used for immunization of mice. Protein was determined spectrophotometrically by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard (Sharma and Banyal, 2009).

Immunization of mice: Five groups of ten mice each; P-II, P-III, S-III, HOM and placebo controls were immunized. Each mouse was injected intraperitoneally with 100 μg of parasite material (placebo controls received no parasite material) and 30 μg of saponin on day 0, 14 and 28.

Collection of sera and challenge of mice: A week after the last immunization dose some of the mice in each group were sacrificed to collect prechallenge immune sera. Sera were stored at -20°C for further use. The remaining mice were inoculated intraperitoneally with 1×10^5 *P. berghei* infected erythrocytes. Course of infection was monitored daily by blood smear examination till the death of animals or clearance of parasite in smear. Sera of the mice which survived the challenge were collected as post challenge immune sera.

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 according to the method of Banyal and Inselburg (1985) as given in Sharma and Banyal (2009).

Indirect flourescent antibody test (IFA): Pre challenge immune sera were also tested by Indirect Flourescent Antibody test (IFA) according to Sharma and Banyal (2009).

SDS PAGE and immunoblotting: The total parasite homogenate and PIII fraction were resolved by SDS-PAGE using 3% stacking and 10% separating gel (Sharma and Banyal, 2011). The separated proteins were blotted onto Nitro Cellulose Membrane (NCM) and incubated with prechallenge sera of mice immunized with P-III and HOM followed by incubation with goat anti-mouse IgG peroxidase conjugated sera (Genei, Banglore) as secondary antibody. Antigen-antibody reactions were detected using 0.05% (w/v) diaminobenzidine tetrahydrochloride (DAB) in 0.01 M PBS, pH 7.2 and hydrogen peroxide.

Immunoadsorption: The different antigenic proteins from the pellet at 24000 g (PIII) were isolated by antibody immobilization method of Hudson and Hay (1989). Known amount of antibody to be coupled was mixed with equal volume of PBS, pH 7.2 and added to an equal amount of CNBr activated Sepharose 4B with gentle mixing and was left overnight at 4°C. Protein of uncoupled antibody was measured spectrophotometrically (A_{280}) and remaining active sites on Sepharose 4B were blocked by treating the gel with 1 M ethanolamine. The uncoupled antibody was 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, pH 7.2 the antibody immunosorbent was poured onto a glass column upto a height of 8 cm at 4°C. The column was pre-eluted with eluting buffer (0.1 M glycine-HCl, pH 2.6; sodium chloride, 0.15 M) and reequlibrated with PBS, pH 7.2. The antigen solution will then be allowed to flow through the column at a speed of 20 mL h^{-1} under gravity. The bound antigens were then eluted with the eluting 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°C till further use. The molecular weights of the purified antigens through immunoadsorption were determined by SDS-PAGE.

RESULTS

Prechallenge immune sera was collected a week after last immunization dose. Six mice from each group were sacrificed to collect sera. Prechallenge sera from rest of the mice were collected from the tail tip. The level of antiparasite antibodies in the prechallenge immune sera of immunized mice was measured by ELISA. Alongwith the experimental group mice, reference malaria positive and reference malaria negative sera were also used. The antibody titres for the prechallenge immune sera of HOM and PIII groups were very high (1:8192). The antibody titre for SIII group was 1:4096. However, PII group showed very low antibody titres of 1:512 (Fig. 1). Serum from placebo control again showed no reaction.

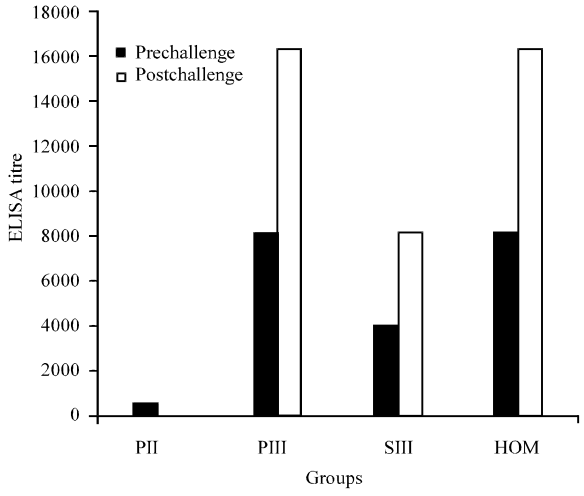

Fig. 1: Level of antiparasite antibodies of mice immunized with HOM, PII, PIII and SIII fraction, in prechallenge immune sera collected on day 35 and post challenge immune sera collected on day 60 post immunization

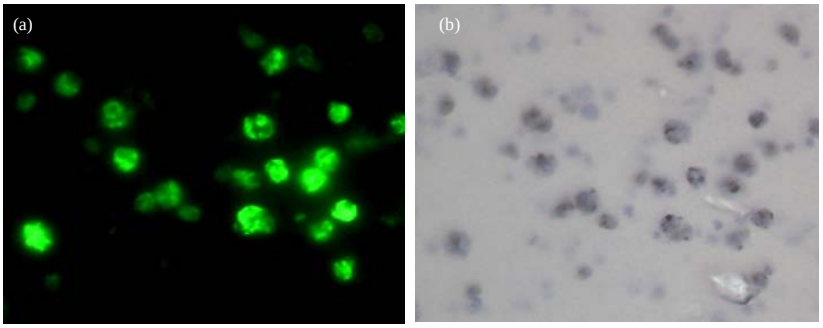

Fig. 2: IFA test using pooled prechallenge sera of P-III group as seen under (a) UV light and (b) Phase contrast (X 1000)

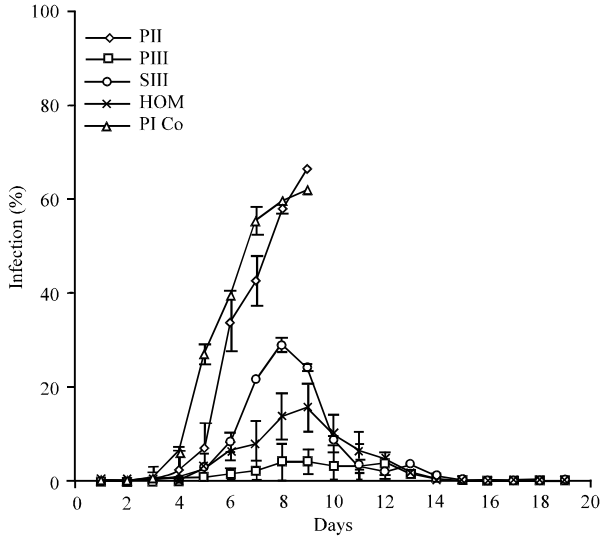

Fig. 3: Course of parasitaemia in mice immunized with total parasite and various subcellular fractions of *Plasmodium berghei* and subsequently challenged with 1×10^5 *P. berghei* infected red cells. Bar represents mean standard deviation

The analysis by IFA revealed positive antigen-antibody reactions. The reaction was specific showing the presence of antiparasite antibodies in the prechallenge immune sera of HOM and PIII groups (Fig. 2), whereas no reaction was seen for PII group and placebo control. SIII group showed weak reaction.

The remaining mice of these groups were challenged with 1×10^5 *P. berghei* infected erythrocytes. Course of parasitaemia in these mice is depicted in Fig. 3. All the mice of placebo control group and PII group died because of infection. Mice in HOM and PIII groups survived the challenge and no parasite was seen in the blood smear by 14th day postchallenge. Maximum parasitaemia in HOM group was 20.1% whereas in PIII group it remained 11.3%. One out of four mice in SIII group survived challenge after having a high parasitaemia of about 29%. Rest of the mice in SIII also died upon challenge.

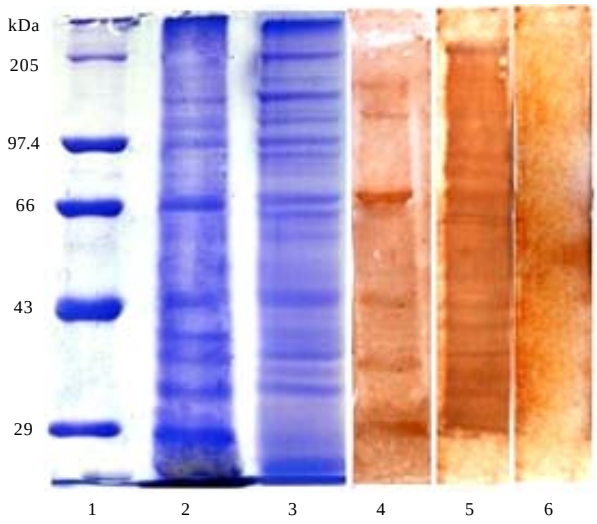

Fig. 4: SDS-PAGE and western blot of different subcellular fractions of *P. berghei*. Lane 1: Protein Standard (SDS-PAGE), Lane 2: P-III (SDS-PAGE), Lane 3: HOM (SDS-PAGE) and Lane 4, 5 and 6 shows immunoblot analysis of prechallenge immune sera obtained from mice immunized with P-III fraction, HOM fraction and Placebo control, respectively

Post challenge immune sera of mice which survived upon challenge were collected and subjected to ELISA. High antibody titres of 1:16384 were recorded for HOM and PIII groups (Fig. 1). However, one mouse of SIII group which survived after challenge showed antibody titre of 1:8192.

Since, the PIII and HOM fractions showed maximum protection and antibody titres therefore, these fractions were analysed further by SDS-PAGE and immunoblotting. The SDS-PAGE of P-III and HOM fractions is shown in Fig. 4. Prechallenge immune sera obtained from mice immunized with P-III were pooled and this pooled serum recognized six antigen proteins of molecular weight 147, 105, 66, 43, 36 and 29 kDa by immunoblotting. Whereas, pooled serum of mice immunized with HOM recognized 17 antigen proteins of molecular weight 205, 182, 171, 165, 137, 112, 94, 78, 66, 61, 53, 46, 43, 40, 36, 32 and 29 kDa (Fig. 4). No band appeared during immunoblotting with the serum obtained from placebo control mice.

Further analysis of prechallenge sera from PIII immunized mice by immunoadsorption revealed that proteins of molecular weights 29, 32, 35, 66, 43, 105, 112 and 147 kDa were immunogenic. However, 66 and 43 kDa proteins were predominantly present in all the fractions.

DISCUSSION

The immunity to malaria involves activation of both humoral as well as cellular immune responses (Singotamu *et al*., 2006). Present study was carried to evaluate the humoral immune response directed against the different subcellular fractions of *Plasmodium berghei*. It is imperative that development of effective antimalarial vaccines rely on identification of critical antigens that elicit strong protective immune responses (Osamor, 2010). Incidentally, success in experimental vaccination against blood stages of malaria parasite does not solely depend on the suitability of the antigen. In fact, factors such as mode of delivery of antigen, route of administration, use of suitable adjuvant etc., play equally important role to induce desired immunological responses.

PIII sediment of *P. berghei* obtained after centrifugation at 24,000 g exhibited a strong humoral immune response in mice. Also, the mice immunized with this fraction showed *in vivo* protection against malaria infection with a very low parasitaemia. Humoral immunity was evident from the high antibody titres in the pre and post-challenge sera as determined by ELISA and IFA. The sera collected from the mice immunized with other subcellular fractions like PII and SIII showed low antibody titres. Mice immunized with PII were not protected when challenged with live parasite and SIII fraction also showed very little *in vivo* protection.

PIII isolated by sedimentation at 24,000 g corresponds to a fraction which contains organelles containing strong hydrolytic enzymes (Banyal *et al*., 1979). Parasite utilizes proteolytic enzymes for the degradation of haemoglobin as well as invasion of erythrocytes (Banyal *et al*., 1981; Sharma *et al*., 2005; Mishra *et al*., 2006; Muller *et al*., 2010; Li *et al*., 2012). Presence of these enzymes in PIII fraction might have a role in inducing antibodies which inhibit propagation of *Plasmodium in vivo*. This is possible because various earlier studies have demonstrated the role of proteases in imparting protection against the disease. Makkar *et al*. (1995) have shown partial protection in mice immunized with acid protease from *Plasmodium berghei*. Falcipain 1, a cysteine protease, of *P. falciparum* is revealed as a vaccine candidate (Kumar *et al*., 2007).

Further immunoadsorption of PIII fraction showed certain antigens particularly of molecular weights 66 and 43 kDa, as revealed by SDS-PAGE, may perhaps be potent in exhibiting *in vivo* protection against malaria. Previous studies on various other species like *P. yoelii*, *P. knowlesi* and *P. falciparum* have also shown partial or complete protection against experimental malaria by immunization with antigens of molecular weights 66 and 43 kDa (Deans *et al*., 1988; Remarque *et al*., 2008).

Perhaps the malaria vaccine has not become feasible because of species and stage specificity in *Plasmodium*. Therefore, antigens which are common in different species and stages if exhibit protection may be the only source of protective vaccine against malaria. Hence, these antigens must be investigated further to determine their role as candidates for an efficacious malaria vaccine.

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REFERENCES

Amiruddin, R., D. Sidik, A. Alwi, N. Islam, Jumriani, P. Astuti and Syafruddin, 2012. Socioeconomic factors and access to health services for malaria control in Mamuju District, West Sulawesi Indonesia. Asian J. Epidemiol., 5: 56-61.

Ayeni, A.O., 2011. Malaria morbidity in Akure, Southwest, Nigeria: A temporal observation in a climate change scenario. Trends Applied Sci. Res., 6: 488-494.

Banyal, H.S., V.C. Pandey and G.P. Dutta, 1979. Subcellular fractionation and localization of marker enzymes of erythrocytic stages of *Plasmodium knowlesi*. Ind. J. Parasitol., 3: 9-14.

Banyal, H.S., G.C. Misra, C.M. Gupta and G.P. Dutta, 1981. Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in plasmodium knowlesi infections. J. Parasitol., 67: 623-626.

Banyal, H.S. and J. Inselberg, 1985. Isolation and characterization of parasite-inhibitory *Plasmodium falciparum* monoclonal antibodies. Am. J. Trop. Med. Hyg., 34: 1055-1064.

Banyal, H.S. and N. Elangbam, 2006. Immunological perspectives and malaria vaccine. J. Parasitic Dis., 30: 16-29.

Deans, J., A. Knight, W. Jean, A. Waters, S. Cohen and G. Mitchell, 1988. Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. Parasite Immunol., 10: 535-552.

Douradinha, B. And D.L. Doolan, 2011. Harnessing immune responses against *Plasmodium* for rational vaccine design. Trends Parasitol., 27: 274-283.

Hudson, L. and F.C. Hay, 1989. Affinity Techniques for Molecules and Cells. In: Practical Immunology, Hay, F.C., O.M.R. Westwood and P.N. Nelson (Eds.). 3rd Edn., Blackwell Scientific Pub., London, pp: 322-329.

Ibrahim, M.A. A.B. Sallau, A. Salihu and K.C. Onwube, 2011. Partial characterization of phospholipase A_2 from the erythrocytic stage of *Plasmodium berghei*. Asian J. Biochem., 6: 208-213.

Kapoor, G. and H.S. Banyal, 2011a. *Plasmodium berghei* thioredoxin reductase induces humoral response in mice. Asian J. Biol. Sci., 4: 96-100.

Kapoor, G. and H.S. Banyal, 2011b. Purification and characterization of *Plasmodium berghei* thioredoxin reductase. Asian J. Anim. Sci., 5: 145-152.

Kumar, A., K. Kumar, R. Korde, S.K. Puri, P. Malhotra and V.S. Chauhan, 2007. Falcipain-1, a *Plasmodium falciparum* cysteine protease with vaccine potential. Infect. Immun., 75: 2026-2034.

Li, H., M.A. Child and M. Bogyo, 2012. Proteases as regulators of pathogenesis: Examples from the Apicomplexa. Biochim. Biophys. Acta, Proteins Proteomics, 1824: 177-185.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Makkar, S., Upma and H.S. Banyal, 1995. *Plamodium berghei* acid proteases elicit antibody response and partially protect mice against infection. J. Parasit. Dis., 19: 123-126.

Mia, M.S., R.A. Begum, A.C. Er, R.D.Z.R.Z. Abidin and J.J. Pereira, 2012. Burden of malaria at household level: A baseline review in the advent of climate change. J. Environ. Sci. Technol., 5: 1-15.

Mishra, R., B. Mishra and N.S.H.N. Moorthy, 2006. Dihydrofolate reductase enzyme: A potent target for antimalarial research. Asian J. Cell Biol., 1: 48-58.

Muller, I.B., J. Knockel, M.L. Eschbach, B. Bergmann, R.D. Walter and C. Wrenger, 2010. Secretion of an acid phosphatase provides a possible mechanism to acquire host nutrients by *Plasmodium falciparum*. Cell. Microbiol., 12: 677-691.

Osamor, V.C., 2010. Global initiatives and local efforts towards management of malaria in Africa. Biotechnology, 9: 196-203.

Remarque, E.J., B.W. Faber, C.H. Kocken and A.W. Thomas, 2008. Apical membrane antigen 1: A malaria vaccine candidate in review. Trends Parasitol., 24: 74-84.

Sharma, A., A. Eapen and S.K. Subbarao, 2005. Purification and characterization of a hemoglobin degrading aspartic protease from the malarial parasite *Plasmodium vivax*. J. Biochem., 138: 71-78.

Sharma, S.K. and H.S. Banyal, 2009. G-glutamylcysteine synthetase from *Plasmodium berghei*. Parasitol. Int., 58: 145-153.

Sharma, S.K. and H.S. Banyal, 2011. Characterization of *Plasmodium berghei* glutathione synthetase. Parasitol. Int., 60: 321-323.

Singotamu, L., R. Hemalatha, P. Madhusudhanachary and M. Seshacharyulu, 2006. Cytokines and micronutrients in *Plasmodium vivax* infection. J. Med. Sci., 6: 962-967.