ABSTRACT

Malaria is no longer a disease of the developed and the developing nations. The impact of this disease is being felt globally, but most seriously in tropical and subtropical areas. Exactly a century after Sir Ross’s Nobel Prize winning discovery that human malaria is transmitted by mosquito, efforts to control the disease have failed and the disease has re-emerged in catastrophic form and causes 1.5-2.7 million deaths each year. Around 300-500 million people get infected with the disease and a 1/3rd of the global population lives in malaria endemic areas. India has faced four major epidemics in the past three years and 2.85 million cases with 3000 deaths were reported in the last year. Although *P. falciparum* is primarily responsible for most of the mortality cases, *P. vivax* affects as many as 35 million people every year. Of all the malaria cases in India, 60-70% is due to *P. vivax* infection. New control measures and treatment are immediately needed to level the disease. A multi-approach research strategy is essential, like vaccine development, genetic engineering of mosquitoes to make them refractory to the development of the sexual stage of the parasite, better understanding of the complex interactions between malaria transmission rates, acquired immunity and the clinical forms and incidence of the disease.

Both mice and humans can be protected from malaria by immunization with irradiated sporozoites. The evidence from genetic and immunological studies in humans and mice that MHC class I restricted CD8+ T cells act as the major effectors mediating a protective immune response has led to attempts to identify preerythrocytic antigen(s), targets of protective cellular responses. A bewildering sequence of recognition, adhesion and invasion events take place in the progression of the life cycle of *Plasmodium*. The parasite must possess a multitude of cell surface ligands/receptors to perform these events. The thrombospondin related adhesive protein (TRAP) is a sporozoite surface protein, proposed to contribute to hepatocyte recognition and invasion. TRAP is a multi-functional, multi-stage parasite antigen, and relatively conserved across the species. Particularly, putative functional domains are conserved and maintained across the species along with positionally conserved cysteine residues. PfTRAP is a target of CD8+ T cells in humans.
living in endemic areas and humans immunized with irradiated sporozoites. This together
with the fact that murine homologues of PfTRAP, PyTRAP and PbTRAP protect mice in a
CD8+ T cell /CD4+ T cell and INF-γ dependent manner, support the vaccine candidacy of
TRAP. Moreover, induction of CTLs specific to conserved epitopes in PfTRAP has been
observed in human volunteers and humans living in endemic areas. Induction of TRAP
specific humoral response in humans living in endemic areas is associated with low
parasite densities. This together with the facts that antibodies to PfTRAP completely
inhibit sporozoite binding/invasion of HepG2 cells in vitro and anti-sulfatide binding motif
antibodies inhibit merozoite invasion of erythrocytes, provides additional evidence to
further substantiate inclusion of TRAP in a multi-component vaccine against human
malaria.

However, lack of suitable animal model is one of the biggest hurdles in assessment
of the protective efficacy and immunogenicity of a candidate antigen. One solution to the
problem is to study an experimental model, which can closely mimic human malaria
parasite in all its biological and molecular aspects such as the P. cynomolgi-rhesus monkey
model. P. cynomolgi bastianelli is a natural parasite of rhesus monkeys (Macaca mulatta)
and serves as a perfect model for the human malaria parasite P. vivax. Both P. vivax and P.
cynomolgi are very closely related in biology, phylogenetic and clinical course of infection.
In addition to this, non-human primate models are susceptible to many species of the
Plasmodium and have immune responses similar to those of humans.

The main objectives of the present study are:

A. 1. Isolation, cloning and sequencing of the TRAP gene from Plasmodium cynomolgi
bastianelli.
2. Expression of P. cynomolgi TRAP (PcTRAP) and its relevant fragments in E. coli.
3. Purification of recombinant proteins.
4. Immunogenicity of recombinant PcTRAP and its relevant fragments in mice.
5. Assessment of the vaccine potential of PcTRAP in rhesus monkeys.
B. 1. Isolation, cloning, and sequencing of Plasmodium falciparum TRAP (PfTRAP).
2. Expression of PfTRAP and its relevant fragments in E. coli.
4. Sero-reactivity of recombinant PfTRAP and its fragments with human samples from endemic areas.

Characterization of *P. cynomolgi* TRAP will be a direct evidence for evaluation of its human counterparts. Expression of the PcTRAP, PfTRAP and their relevant fragments in *E. coli* will provide reagents to assess immunogenicity of TRAP in experimental animals and naturally infected humans, respectively. Furthermore, this study will also answer whether some functional regions of the TRAP remain cryptic or have some immunological relevance for the parasite. The experimental methodology used to achieve these objectives and the results obtained are reported in the different chapters of this thesis.

The literature regarding malaria and TRAP has been reviewed in chapter 1. Isolation and sequence analysis of the TRAP gene from *P. cynomolgi bastianelli* has been described in chapter 2. The PcTRAP gene was amplified from PcgDNA using primers based on conserved regions. Sequencing of the cloned PCR fragment revealed a 1638 bps sequence with 55.56% AT content. Analysis of this 1638 bps sequence revealed that it represents an ORF of 1635 bps, which ends as the termination codon TAA (1636-1638). Translation product of the gene corresponds to a 545 amino acid polypeptide with a predicted molecular mass of 59.68 kDa. Analysis of the amino acid composition indicated acidic nature of the protein (pI 4.45, acidic amino acids 17.06%) with high asparagine (8.42%) and proline (9.89%) contents. There are 11 cysteine residues in the molecule. Hydropathy analysis showed presence of a hydrophobic domain (aa. 483-512) followed by a cytoplasmic tail (aa. 523-545) at the C-terminus of the molecule. PCGENE analysis of the deduced sequence showed presence of an integrin like magnesium binding A-domain (~200 amino acid residues), a thrombospondin like domain (WTPCSVTCGKGTSHSRSR) and an amino acid run (a stretch of six aspartic acid residues).

Alignment of the amino acid sequence of PcTRAP with that of other TRAP molecules from *P. vivax*, *P. knowlesi*, *P. falciparum*, *P. berghei*, *P. yoelii*, and *P. gallinaceum* showed that PcTRAP shares structural elements common to other TRAP molecules (Fig. 2.5). This basic structural organization contains hydrophobic domains at both ends, a ~200 amino acid long integrin like magnesium binding domain (A-domain or I-domain), a thrombospondin like sulfatide binding motif (region II in CS proteins), an asparagine - proline rich divergent region with short repeats, and a cytoplasmic tail at
extreme carboxy terminal end. Conserved sequences include sequence near signal peptide cleavage site, DEIKYSEEVC, magnesium ion dependent adhesion sites (DXSXS, T, D), sulfatide binding domain, transmembrane domain and cytoplasmic tail. Most significantly all the 11-cysteine residues are positionally conserved across the species. The divergent region lacks conserved sequences and rather varies in length among TRAP molecules due to presence of repeats. PcTRAP does not contain significant repeats as seen in PyTRAP, PbTRAP and PgTRAP. Repeats are rich in proline and aspartagine residues; however, their length number and sequence vary among TRAP molecules.

Furthermore, a comparison of percent homology of PcTRAP with other TRAP molecules suggested that it is the closest to PvTRAP (73.9%) followed by PkTRAP (71.9%).

**Chapter 3.** Cross species comparison revealed that TRAP has highly structured multi-domain organization with multiple putative functional domains. These structural features are organized in such a way that all the functional domains (A-domain and sulfatide binding domain) and 10 positionally conserved cysteine residues form the N-terminal half of the exodomain. Whereas, aspartagine-proline rich acidic divergent region forms C-terminal half of the exodomain. Keeping this structural vs. functional relationship and conserved/divergent vs. immunological importance of the domains, three fragments of *PcTRAP* gene were expressed in *E. coli*. These were PcTRAP-F (represents complete exodomain aa. 1-483), PcTRAP-N (represents N-terminal half of the exodomain which contains A-domain, sulfatide binding domain, and 10 positionally conserved cysteine residues, aa. 1-269) and PcTRAP-C (represents aspartagine–proline rich acidic divergent region of exodomain aa. 270-483). Expression of these as an N-terminal fusion of 6xHistag (PcTRAP-F and PcTRAP-N), an N-terminal fusion of MBP (PcTRAP-C), and a C-terminal fusion of 6xHistag (PcTRAP-C_His) facilitated one step purification or enrichment of these proteins by nickel-chelate affinity chromatography or by amylose affinity chromatography.

The vaccine grade PcTRAP-F was produced by first step purification by Ni-NTA affinity chromatography and second step purification by anion-exchange chromatography using FPLC. As low molecular weight impurities present in Ni-NTA purified PcTRAP-F could not be removed under native condition, the second step purification was carried out
under denaturation condition. Removal of these impurities was possible when βME and Triton X-100 were present in the buffer, suggesting that impurities might be associated with PcTRAP-F by strong hydrophobic interaction and by disulfide bonds.

The inclusion body extract containing PcTRAP-N did not bind to Ni-NTA resin. Whereas the flow through containing PcTRAP-N bound to Ni-NTA resin. This could be due to the presence of inhibitory substances in the inclusion body extract, which are removed upon Ni-NTA adsorption. Therefore, large-scale purification of PcTRARP-N involved prior adsorption of inclusion body extract on Ni-NTA resin. Expression of PcTRAP-C as an N-terminal MBP fusion protein certainly gave excellent yields of MBP/PcTRAP-C fusion protein, however, did not bind well to amylose resin. Therefore, a 6xHis-tag was inserted in the C-terminal of PcTRAP-C, which facilitated purification by Ni-NTA affinity chromatography under native condition. This protein was further purified by anion exchange chromatography.

Immunogenicity of the recombinant PcTRAP fragments was determined in mice and protective potential of the recombinant PcTRAP-F was assessed in rhesus monkeys against *P. cynomolgi* sporozoite challenge. As determined by ELISA, it appears all the recombinant forms of the PcTRAP are highly immunogenic. Our immunization data with two strains of mice and monkeys suggests that immunogenicity of the PcTRAP-F is not at all influenced by the genetic background of the animal immunized. Surprisingly after priming with PcTRAP-F or PcTRAP-F_red-alkyl (reduced and alkylated) whole antibody response was directed towards C-terminal region of the PcTRAP-F, which was switched over to N-terminal region upon subsequent boosts. This diversion of immune response towards C-terminal region in mice after the first immunized with PcTRAP-F appears to be due to the presence of divergent region, as mice immunized with PcTRAP-N (N-terminal region) showed eight-fold higher antibody titer to PcTRAP-N. Diversion of immune responses toward variable/repeat regions has been thought to be a strategy of the malaria parasite to evade the host immune system. The observation that mice immunized with PcTRAP-F_red-alkyl developed eight-fold antibody response against N-terminal suggests that the poor antibody response against this region in mice immunized with PcTRAP-F could be partly attributed to disulfide linkages mediated secondary folding of the protein. At the same time higher antibody response against N-terminal region in mice immunized with PcTRAP-F_red-alkyl might be due to presentation of new epitopes, which were cryptic due to disulfide
linkages (there are ten cysteine residues in the N-terminal region). The high antibody response against PcTRAP-C (C-terminal region) in mice immunized with PcTRAP-F, PcTRAP-F_red-alkyl or MBP/PcTRAP-C is consistent with the hydrophilicity of this region as well the observation that variable/repeat region of Plasmodium antigens elicit very high antibody response.

The pre-challenge total antibody responses in monkeys immunized with recombinant PcTRAP-F were not as high as in mice. Consistent with the mice data, antibody response was primarily directed towards C-terminal region. Of the three immunized monkeys; one (8989) showed sterile immunity against the sporozoite challenge, one monkey (9086) showed delayed as well as lower peak parasitemia and one monkey (8990) developed parasitemia, like controls. However, one adjuvant control monkey (9076) also showed delayed and lower peak parasitemia. The partially protected immunized and adjuvant control monkeys did not show second peak parasitemia as observed with the control monkeys. Our protection results are in agreement with the fact that a pre-erythrocytic vaccine should provide sterile immunity as well as reduce blood stage parasitemia by reducing the number of the infecting sporozoites. However, partial protection status of one of the adjuvant control monkey appears to be due to adjuvant mediated nonspecific immunity. Correlation of antibody response towards N-terminal region with protection implies that antibodies directed against functional regions are protective rather than non-protective high antibody response against divergent/repeat regions.

The chapter 4 of the thesis is related to production of recombinant PfTRAPs to analyse TRAP specific humoral response in people exposed to malaria. To achieve this PfTRAP gene was amplified from the PfDNA and sequencing of the gene showed remarkable similarity with published T9/96 sequence. To study the humoral response to TRAP under natural conditions and to analyse possible association between this response and to susceptibility to malaria, three PfTRAP fragments were chosen for expression in E. coli. These recombinant proteins represent full-length exodomain (PfTRAP-F, aa. 26-500), putative functional region of the exodomain (PfTRAP-N, aa. 26-310), and the divergent/variable region of the exodomain (PfTRAP-C, aa. 316-500) (Fig. 4.1). All the three recombinant PfTRAPs were purified in soluble form (in PBS). The recombinant proteins were used in ELISA to analyse antibody responses.
Recombinant TRAP and its N and C-terminal fragments were recognised by individuals from areas of different endemicity in ELISA and immunoblot. The proportion of IgG responders to recombinant C-terminal protein increased with malaria endemicity with Nigerian sera showing highest recognition of 63.8% followed by Kalta (41.7%), while it was only 27.7% in samples tested from Rourkela region. There were no significant differences between median levels of IgG to PfTRAP-F between different areas of endemicity. The levels of IgG response to different recombinant PfTRAP fragments and parasite lysate were not significantly different among donors of the different age groups. However, further studies are needed to clarify the relative importance of the N-terminal TRAP region vis-a-vis more polymorphic C-terminal fragment. In conclusion, this field investigation on the immune responses directed against TRAP has shown that it is naturally immunogenic and the IgG reactivity increase with malaria endemicity. At the same time evaluation of TRAP specific cellular responses in humans living in endemic areas appears equally important as cellular responses have been implicated in protection against sporozoite infection.

In conclusion this study showed that PcTRAP is the closest to PvTRAP, therefore substantiated the use of *P. cynomolgi*-rhesus monkey model for evaluation of vaccine candidate antigens. Furthermore, recombinant PcTRAP is capable of inducing immune response independent of genetic restriction and humans living in endemic areas develop TRAP-specific humoral response. All these together with the fact that recombinant PcTRAP elicits protective response in rhesus monkeys against sporozoite challenge, will be of interest in the development of a human malaria vaccine.