Surinder Kumar (2011). ***Immunological and functional characterization of putative target(s) in Malaria.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.

Supervisor: Dr. G.C. Varshney **Acc. No. : Th-253**

SUMMARY OF THE THESIS

The asexual blood cycle of Plasmodium parasite is responsible for all the symptoms that are associated with infection, and vaccine-induced immunity against asexual blood stages has potential to attenuate the symptoms and complications of the disease even if protection is not complete. Therefore, the molecules which are associated with asexual blood stage (involved either in host cell invasion or modification) are most compelling targets for anti-malarial drugs and vaccine development. The components from rhoptries have been implicated in merozoite invasion, formation of the parasitophorous vacuole, and subsequent growth of the parasite within erythrocyte. Among plethora of rhoptry associated molecules, RhopH3 appears to be essential for asexual development as judged from unsuccessful attempts to disrupt its gene locus. Further, strong immune response against RhopH3 has been observed in malaria-infected people and anti-RhopH3 antibodies are able to inhibit merozoite *in vitro* invasion of red blood cells (RBC). However, characterization of potential protective epitopes in the conserved regions and their immunogenicity is necessary to better evaluate the potential of RhopH3 as a vaccine candidate against blood-stage malaria. Further, a number of parasite-encoded proteins exported into and through the host erythrocyte are responsible for modifications both to erythrocyte cytoskeleton and the extra cellular face of the membrane. These infected cell surface determinants have been the focus of extensive research for developing therapeutics as well as for generation of effective immunity against disease. However, majority of the antigens identified so far are antigenically diverse or polymorphic in nature, which is a great problem in targeting them for vaccine design. Thus, identification of structurally/functionally conserved unique molecules, associated with infected cell or parasite surface is highly warranted.

Multiple sequence alignment of RhopH3 protein from various species of Plasmodium revealed the clustering of long stretches of conserved domains in N-terminus and central region while C-terminal region was highly variable. Further, the prediction of putative B-cell epitopes in the conserved domains of Plasmodium RhopH3, by B-cell prediction tools such as ABCpred and BepiPred, revealed the importance of N-terminus (aa 1-147) and central region (aa 474-657) of the RhopH3.

During over expression studies of N-terminus (aa 1-147) and central region (aa 474-657) of the RhopH3, expression of central region could not be observed in *E. coli* BL21 (DE3) host cells. Considering AT biasness of Plasmodium gene and different codon

preference as compared to *E. coli*, expression was checked in codon plus strain. However, very low level of expression could be seen at position of 20.6 kDa (expected size of aa 474-657 region of RhopH3). Further, the expression of rRhopH3$_{474-657}$, when checked in other strains like Rosetta (DE3) pLys, no improvement in expression level of the protein could be seen. Although, *E. coli* BL21 (DE3) cells containing N-terminal region of RhopH3 i.e. rRhopH3$_{1-147}$ gene cloned in pET vector, upon induction with 1mM IPTG, over-expressed 18kDa fragment in accordance with the expected size, yet majority of rRhopH3$_{1-147}$ was found in inclusion bodies. In attempts to purify and refold protein, it was observed that rRhopH3$_{1-147}$ protein was soluble in 8 M urea. The rRhopH3$_{1-147}$ was thus purified under denaturing conditions using Ni-NTA affinity column. The refolding of protein was carried by step-wise dialysis with decreasing the concentration of urea in each step. It was observed that protein retains its solubility in buffer having 1.25 M urea.

Anti-rRhopH3$_{1-147}$ antiserum exhibited good level of reactivity with rRhopH3$_{1-147}$ fragment as well as native protein RhopH3 of both human and rodent malaria parasites. Immunofluorescence studies using anti-rRhopH3$_{1-147}$ antiserum showed characteristic punctuated fluorescence of rhoptries. Further, antiserum recognized 105-110 kDa rhoptry protein of *P. berghei* and *P. falciparum*, indicating that the conserved epitopes are immunogenic. The generated antibodies were also capable of inhibiting the *in vitro* growth of *P. falciparum*. Further, during immunological studies performed, taking rRhopH3$_{1-147}$, following results were obtained:

i) Recombinant RhopH3$_{1-147}$ induced the potent proliferation of lymphocytes, obtained from rRhopH3$_{1-147}$ immunized mice, revealing that it is a good immunogen and has the potential to induce antigen specific T cell response.

ii) Lymphocytes obtained from rRhopH3$_{1-147}$ immunized mice showed dose dependent increase in IL-2 and IFN-γ secretion, thus resulting in Th1 type of T cell response.

iii) Antiserum obtained from rRhopH3$_{1-147}$ immunized mice showed strong IgG2a response, further confirming that rRhopH3$_{1-147}$ induces Th1 type of T cell response.

iv) *In vivo* protection study carried out using *P. berghei* as a model system following immunization with rRhopH3$_{1-147}$ showed significant delay of parasitemia development which demonstrated the vaccination potential of the epitopes contained in this region.

In our earlier lab studies (Kaur, 2008), a panel of 12-mer phage peptides, reactive to mAbB6 (*in vitro P. falciparum* growth inhibitory), was isolated. Clustal X alignment of all these mimotopes, taking together, indicated aa 512-571 region as possible epitope of mAbB6. In this study attempts were made to further dissect the epitopic region(s) of RhopH3 reactive to mAbB6. Blast search showed that peptides R1p4, p6, p9, p11 and p16, isolated using mAbB6, have 42%, 67%, 58%, 42% and 17% sequence homology respectively to *P. berghei* RhopH3. Based on blast search homology, clustal X alignment and correlation with predicted B-cell epitopes, the regions of RhopH3; 673-686, 512-551, 699-732 and 66-82 aligning with phage peptides; R1p1, R1p4, R1p11 and R1p16 respectively were selected for further experimentation in order to identify mAbB6 reactive epitopic region(s). Competitive inhibition and SPR analysis indicated that aa 66-82 region of RhopH3 corresponding to phage peptide R1p16 may constitute a region of mAbB6 reactive epitope. This was further substantiated by observation that this region reside in the loop region having surface accessibility and is conserved among different species of malaria parasite.

Mimotopes, reactive to mAbB6, displaying different levels of antigenicity were checked for their immunogenic potential. Antisera raised against phage peptides R1p1, R1p6, R1p9 and R1p16; i) reacted well with their corresponding phage peptides, ii) exhibited mAbB6 like reactivity, in western blotting, with affinity purified RhopH3, iii) reacted well with *P.berghei* extract in ELISA and, iv) recognized *P.berghei* infected cell surface in immunofluorescence study. Further, these sera also reacted with *P.falciparum* extract, thus further validating conserved nature of epitope recognized by mAbB6. Moreover, like original mAbB6, anti-mimotopes antisera exhibited parasite growth inhibitory potentials thereby mimicking the functional epitope of mAbB6. These results conclusively demonstrated that phage peptides; R1p1, R1p6, R1p9 and R1p16 are the antigenic as well as immunogenic mimics of the epitope recognized by mAbB6.

In second part of this study, while searching for structurally/ functionally conserved (novel) molecules associated with the parasite and infected cell, attempts were made to identify and characterize the molecule(s) by taking selected monoclonal antibody (mAb). Among panel of monoclonal antibodies generated, mAbB9A10 was selected based on; i) its cross reactivity with both *P. falciparum* & *P. berghei* parasites & infected cells (in search of conserved molecules) and, ii) *in vitro P. falciparum* growth inhibitory activity.

This antibody (mAbB9A10), which was found to be of IgG2b isotype, was taken further to identify and characterize the molecule(s) as well as the region(s) reactive to this mAb.

Immunoblotting of infected erythrocytes extract with mAbB9A10 showed the presence of two components at positions corresponding to molecular weights 112kDa and 37kDa in case of *P. falciparum* and a single band at position of 66kDa in case of *P. berghei*. Affinity purification of 112kDa molecule, using mAbB9A10 affinity column resulted in poor yield which hampered its detailed characterization. Although, few attempts were made, using isolated mimotopes, to identify 112kDa component. These results have been summarized in later part of the summary. On the other hand, 37kDa component could be obtained in affinity column eluate. mAbB9A10 reacted well with this affinity purified component. Further, identity of 37kDa component was established by peptide mass fingerprinting and confirmed by LC-MS/MS analysis. The identity of 37kDa component, reactive to mAbB9A10, was established as Glyceraldehyde-3-phosphate dehydrogenase of *P. falciparum* with following observations;

i) 18 peaks from peptide mass fingerprint of 37kDa molecule matched with peptide mass database (deposited in non-redundant database of NCBI using MASCOT search engine) of PfGAPDH with a top score of 207.

ii) LC-MS/MS fragmentation spectra of tryptic products from 37kDa molecule confirmed further that 37kDa molecule recognized by mAbB9A10 is "Glyceraldehydes-3-phosphate dehydrogenase" with a top score of 398.

Further, 37kDa GAPDH band appeared different from 112kDa component, as standard anti-GAPDH (human NRBC) antibody, when probed with PfIRBC extract, recognized only 37kDa component, not the 112kDa component. Secondly, mAbB9A10 doesn't cross react with human GAPDH (from NRBC), signifying the specificity of this antibody to parasitic component(s) only.

The screening of random peptide phage library with mAbB9A10 yielded many different peptides with following observations;

i) Among all peptides, the peptide A10p4 (ATWSHHLSSAGL) was found in 7 out of 17 clones indicating its sequence preference for binding to mAbB9A10.

ii) A motif "SXHL" was found in majority of the sequences (including A10p4) specific to mAbB9A10, where X is any amino acid, indicating that the motif

"SXHL" may constitute the epitope or part of the epitope reactive to mAbB9A10.

iii) The mimotope A10p4 upon blast search retrieved a rhoptry protein i.e. rhoptry neck protein 6 (RON6: PFB0680w) with 60% identity, corresponding to molecular weight of 112 kDa.

iv) The motif "SXHL" is also present in rhoptry neck protein 6 and aligned in the aa 741-750 region of *P. falciparum* RON6 sequence. Thus, it is likely that mAbB9A10 reactive 112kDa molecule may be PfRON6.

Interestingly, Clustal X alignment showed the presence of "SXHL" motif in 109-112 amino acid region of GAPDH. Moreover, inhibition of the binding of mAbB9A10 to native antigen in parasite extract by "SXHL" domain containing regions of RON6, PfGAPDH and peptide mimotope A10p4 may explain the observed cross reactivity of mAbB9A10 and simultaneously indicate that this domain may constitute the part of the epitope recognized by mAbB9A10.

Overall, the important findings of the current study are:

1. Immunizations with rRhopH3$_{1\text{-}147}$, from N-terminus of the RhopH3, induced Th1 type of immune response and resulted in the significant delay of *P. berghei* parasitemia development *in vivo*. Further, anti- rRhopH3$_{1\text{-}147}$ antiserum also inhibited *in vitro* growth of *P. falciparum*.
2. Amino acids 66-82 region of RhopH3 might constitute a part of an epitope (may be conformational) reactive to growth inhibitory mAbB6. Phage peptides, reactive to this mAb, were found to be antigenic as well as immunogenic mimics of the epitope on RhopH3.
3. *In vitro P. falciparum* growth inhibitory mAbB9A10 recognizes parasite glyceraldehyde-3-phosphate dehydrogenase (37kDa) and possibly cross reacts with rhoptry neck protein 6 (112kDa) of *P. falciparum*.

Therefore, N-terminus of RhopH3 and the identified epitope/mimotopes reactive to mAbB6 may be suitable for the development of anti-malaria vaccine. Also, characterization of the components, identified in this study, using parasite growth inhibitory mAbB9A10, might be useful in understanding their role in parasite survival as well as for exploitation as drug/vaccine target.