SUMMARY

The malaria parasite invades and infects an erythrocyte during the asexual stage of its life cycle, where it extensively modifies the host erythrocyte membranes. These alterations appear to be obligatory for the parasite development. Plasmodial proteins exported to the erythrocyte membrane are thought to mediate nutrient uptake, ionic balances and cytoadherence. Although, the mechanisms underlying these infection-induced changes in the IRBC are not precisely known, it has been suggested that the parasite-synthesized proteins directed at the host erythrocyte membrane actuate such observed phenomenon. These infected cell surface determinants have been the focus of extensive research since the past few decades in understanding the mechanisms of pathogenesis as well as for developing immunodiagnostics and therapeutic interventions. In addition, the infected host cell membrane has been an obvious target for differential drug action because it provides various new routes for the bi-directional traffic of materials needed for parasite subsistence, some of which could be affected by drugs, while others could serve as access routes for cytotoxic drugs otherwise impermeant to uninfected cells.

In order to identify and characterize these infection-induced determinants, associated with the host cell membrane, a panel of monoclonal antibodies (mAbs) was generated in the previous study in the lab using homologous immunizations with the crude membrane preparation of *Plasmodium berghei*-infected erythrocytes. Two monoclonal antibodies, mAbF10 and mAbD2 were found to react with the infected erythrocyte membrane as well as with the parasite. Further, kinetic studies with these mAbs revealed that unlike mAbD2, mAbF10 exhibited a temperature-dependent uptake in IRBC *in vitro* and selectively delivered drug to the infected erythrocytes *in vivo*. In this study, attempts were made to establish the identity of these mAb-recognized molecule(s) present in the infected cell membrane, to further characterize, and to check the possibility of their involvement in antibody (macromolecular) uptake.

Affinity matrices, prepared using mAbF10-Sepharose isolated components of 230-kDa, 195-kDa and a doublet at 156-kDa. Occasionally, a faint band corresponding to 95-kDa was also observed. Similar affinity purification using mAbD2-Sepharose yielded components of 146-kDa and 105-kDa. These molecules were not detected either in normal red blood cells or in the reticulocytes, thus establishing that these were infection-induced.

The SDS-PAGE analyses of mAbF10-affinity-purified components under reducing and non-reducing conditions were similar, thus ruling out any contribution of disulfide bridges in the association of 230-kDa, 195-kDa and the 156-kDa components. However, apparent shifts in mobility of mAbD2-isolated components were observed, where the migration of 146-kDa and 105-kDa molecules under reducing condition changed to 141-kDa and 116-kDa positions respectively, under non-reducing conditions. Nevertheless, the presence of two distinct moieties under both reducing and non-reducing conditions ruled out any inter-molecular disulfide linkages. Immunoblotting of SDS-PAGE separated components further indicated the recognition of 230-kDa, 195-kDa and 156-kDa (doublet) by mAbF10 under both the conditions. On the other hand, the reactivity of mAbD2 was confined to only 105-kDa/116-kDa molecule. The absence of any recognition of the 146-kDa/141-kDa component indicated it to be a 105-kDa associated moiety.

Amino-terminal sequencing of the individual mAbF10 purified components (230-kDa, 195-kDa and 156-kDa) indicated a common N-terminal of ETIEVYND, thus indicative of being alternate processed products of a common precursor. BLAST searches at ncbi.nlm.nih.gov server, showed 77-100% homology to the variants of the merozoite surface antigen/protein-1 (MSA/MSP-1) of rodent malaria parasites. Additionally, on digestion with *Staphylococcus aureus* V8 protease, the partial proteolytic profiles of all these components showed the presence of a common polypeptide at 23-kDa position, thus confirming that they are related to a common precursor molecule. Accordingly, a nomenclature of MSP-1_{230}, MSP-1_{195} and MSP-1_{156} was assigned to represent the respective component.

The N-terminal sequence (KDYFNGVLNQKL) of the 105-kDa molecule, recognized by mAbD2, followed by homology searches, revealed 93% identity to the high molecular weight rhoptry protein 3 (RhopH3) of *P. yoelii*. However, the second component of 146-kDa could not be sequenced, possibly because of the blockade at its N-terminus. Nevertheless, the contrastingly different profiles, obtained from the limited proteolysis of 146-kDa and the RhopH3 (105-kDa) ruled out any precursor-product relationship.

The MSP-1 and RhopH3 determinants, reactive with mAbF10 and mAbD2, respectively were present in both infected cell membrane and parasite as evident by following results:

- Both the mAbs recognized *P. berghei*-infected erythrocytes in direct immunofluorescence assay (IFA), using air-dried infected red blood cells (IRBC). The reactivity of mAbF10 and mAbD2 was mainly confined to the intracellular parasite, although a faint fluorescence was also observed at the erythrocyte surface. The fluorescence pattern exhibited by mAbD2 was characteristically punctate pattern similar to rhoptry organelle staining. This suggested the presence of the respective determinants recognized by mAbF10 (*i.e.*, MSP-1) and mAbD2 (*i.e.*, RhopH3) on both the parasite and erythrocyte membrane.
- Immunoblots revealed the prevalence of MSP-1_{230}, MSP-1_{195} and MSP-1_{156} and the mAbD2-recognized 105-kDa RhopH3 in both the erythrocyte membrane-rich fraction (MRF) and parasite-rich fraction (PRF).
- Infected cell membranes, affinity-purified using rabbit anti-mouse NRBC antibody-Sepharose column, showed the presence of MSP-1 (MSP-1_{230}, MSP-1_{195} and MSP-1_{156}) and RhopH3 (105-kDa), when probed with mAbF10 and mAbD2, respectively.

[^{35}S] methionine-labeling, followed by immunoprecipitation using protein A-Sepharose beads pretreated with rabbit anti-mouse immunoglobulins, detected the presence of only RhopH3 in the percoll-purified membranes, although both labeled 146-kDa and RhopH3 were observed in the parasite-rich fraction. Similar immunoprecipitations protocol, using mAbF10, failed to detect any labeled component either in PRF or MRF. However, the immunoprecipitation using mAbF10-conjugated Sepharose beads did reveal the labeling of MSP-1_{230}, MSP-1_{195} and MSP-1_{156} in both the MRF and PRF. Labeling of 95-kDa was also seen in the immunoprecipitations from the PRF.

The presence of RhopH3 was seen in both early and later stages of infection, while the 146-kDa component appeared in the mature-stage IRBC. MSP-1_{230}, MSP-1_{195} and MSP-1_{156} were observed in trophozoite-enriched IRBC, while a faint band corresponding to MSP-1_{230} could only be detected in the early-stage infected fraction.

The presence of fungal metabolite brefeldin A (BFA) did not show any variations in the relative intensities of MSP-1_{230}, MSP-1_{195} and MSP-1_{156}, or 105-kDa (RhopH3) and 146-kDa molecules.

Following results were obtained for the studies performed to localize MSP-1 (mAbF10-reactive) and RhopH3 (mAbD2-reactive) on infected cell surface:

- Ultrastructural studies by immunoelectron microscopy at 4°C, using the mAb-nanogold conjugates, showed the localization of the mAbF10-nanogold at the surface-exposed face of the erythrocyte membrane. This surface localization was not a uniform distribution, but concentrated at distinct 'moieties' on the erythrocyte membrane. However, the same for mAbD2 could not be inferred, because of the absence of any observed mAbD2-nanogold particles in IRBC sections, possibly due to alterations during sample processing for electron microscopy.
- Enriched blotting studies of mAb-Sepharose immunoprecipitated components from surface-biotinylated IRBC extract with avidin-HRP showed that all the mAbF10-immunoprecipitated MSP-1_{230}, MSP-1_{195} and MSP-1_{156} were biotinylated. On the other hand, amongst the two mAbD2-immunoprecipitated components, only the 105-kDa (RhopH3) and not the associated 146-kDa, was detected with avidin-HRP. This observation, and the results obtained in [^{35}S] methionine labeling experiment, conclusively demonstrated the absence of 146-kDa molecule in infected cell surface.

The identification of the homologues of MSP-1 and RhopH3, in *P. chabaudi* and *P. yoelii* was carried out using mAbF10 and mAbD2. Immunoblotting with mAbF10 revealed the presence of a 230-kDa molecule in *P. chabaudi* and 230-kDa, 156-kDa and 95-kDa in *P. yoelii*-infected cell. While, mAbD2-reactive component RhopH3 (105-kDa) in *P. berghei* was also detected in *P. yoelii*. However, no reactivity of mAbD2 was evident with *P. chabaudi*. Metabolic labeling of *P. chabaudi* and *P. yoelii*, followed by immunoprecipitation with mAbF10-Sepharose, isolated a 230-kDa molecule in *P. chabaudi* and 230-kDa, 195-kDa, 156-kDa doublets and 95-kDa in *P. yoelii*-infected erythrocytes. Using mAbD2-Sepharose beads, labeled components of 105-kDa and 146-kDa were found to be immunoprecipitated from *P. yoelii*-infected erythrocytes. Neither component was isolated by mAbD2-Sepharose from biosynthetically labeled *P. chabaudi*-infected erythrocytes.

Immunoelectron microscopic studies at a metabolically permissible temperature (37°C) showed a temperature-dependent uptake of mAbF10-nanogold in infected erythrocytes. Intraerythrocytic clusters of mAbF10-gold were found near the vicinity of

the parasite as well as within parasitic compartment. Any such intracellular accumulation was not observed with either mAbD2- or mAb44G3-nanogold conjugates. Inhibitors of caveolae-mediated uptake, did not show any inhibition in the intracellular ^{125}I-mAbF10 accumulation in IRBC. Also, ATP-depletion did not affect the kinetics of ^{125}I-mAbF10 uptake. These results indicated the possible involvement of MSP-1 in specific uptake of mAbF10 by yet unknown mechanism(s).

In limited attempts towards the cloning of the MSP-1 and RhopH3 gene of *P. berghei* NK65, the former could not be cloned because of its large size. While the complete nucleotide sequence of RhopH3 protein of *P. berghei* was determined. Genetic alignments, Clustal W, with the analogues of RhopH3 in *P. yoelii* (*Py*RhopH3) showed an overall identity of 87.8% and a similarity of 94.5%. Alignment with the human malaria parasite *P. falciparum* RhopH3 (*Pf*RhopH3) also showed identities at distinct stretches of blocks, similar to that reported earlier between *Py*RhopH3 and *Pb*RhopH3.

Overall, the important findings of the present study are:

(i) The 105-kDa RhopH3 antigen of *P. berghei* was isolated and its entire cDNA sequence determined. Another molecule of 146-kDa, in association with RhopH3, was also detected in the parasite. While in the infected cell membrane, only RhopH3 was present.

(ii) A monoclonal antibody reactive against *P. berghei*-infected erythrocytes, and effectively internalized, identified MSP-1 on infected cell membrane. Several lines of evidences conclusively demonstrated its presence also on infected cell surface. A possible involvement of MSP-1 in specific uptake of mAbF10, by yet unknown mechanism(s), is suggested.