Research Articles

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Generation and characterisation of monoclonal antibodies specific to *Plasmodium falciparum* enolase

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Abstract

Background & objectives: Glycolysis is the sole source of energy for the intraerythrocytic stages of *Plasmodium falciparum,* making glycolytic enzymes putative therapeutic targets. Enolase, a single copy gene in *P. falciparum* is one such enzyme whose activity is elevated ~10–15 fold in infected RBC's. It holds the possibility of having multiple biological functions in the parasite and hence can be a suitable candidate for diagnostic and chemotherapeutic purposes.

Methods: We have aimed at generating parasite-specific reagents in the form of monoclonal antibodies. We have raised monoclonal antibodies against the recombinant *P. falciparum* enolase.

Results: Two IgG monoclonals were obtained with 1:1000 titre and specific for *P. falciparum* enolase. Apicomplexan parasites including *P. falciparum* enolase has a plant like pentapeptide sequence (^{104}EWGWS^{108}) which is uniquely different from the host counterpart. A peptide spanning this pentapeptide region (ELDGSKN*EWGWS*KSK) coupled to BSA was used to raise parasite-specific antibody. Four monoclonals were obtained with 1:1000 titre and of IgM isotype.

Interpretation & conclusion: All the monoclonals are specific for *P. falciparum* enolase and one of them display reactivity against native *P. falciparum* enolase signifying this pentapeptide to be surface exposed and immunogenic.

Key words ELISA – enolase – monoclonal antibodies – *Plasmodium falciparum*

Introduction

Malaria remains a major threat to public health worldwide. It is estimated that 1–2 million children die each year mostly in sub-Saharan Africa due to severe complications of *Plasmodiun falciparum* malaria[1]. Attempts to reduce the rates of morbidity and mortality have been hampered by the increasingly limited efficacy of current antimalarial drugs to which *P. falciparum* has developed resistance[2]. Renewed efforts are required to develop novel and affordable antimalarials to overcome the detrimental effects of drug resistance, particularly in developing countries[3]. *P. falciparum* which is the causative agent

for most lethal forms of malaria, is known to lack a functional Kreb's cycle in its intra-erythrocytic stages and hence solely rely on glycolysis for its energy needs[4]. The glucose uptake is enhanced between 30 and 100 times greater than that of the uninfected erythrocyte[5] and almost all of it is converted to lactate[6]. The increased metabolic activity of the parasite during this period is reflected in the higher activity levels of glycolytic enzymes compared to those of the erythrocyte[7]. Since parasite has a single gene for enolase and the activity of this enzyme is essential for the operation of glycolysis[8], *Plasmodium* enolase protein can be a potential target for antimalarial drugs. The infected RBC's have ~15–20 fold higher enolase activity as compared to uninfected cells[9] and as enolases are known to be multifaceted proteins with several other non-glycolytic functions[10], it is likely that parasite enolase may also be involved in novel biological function(s). Further, apicomplexan enolases are phylogenetically more closely related to plant enolases than to the mammalian counterparts. Thus parasite enzyme may constitute a novel chemotherapeutic[11] and/or diagnostic[12] target for malaria. With these considerations, we have recently cloned, over expressed and purified *P. falciparum* enolase[13]. Previously, we have raised polyclonal antibodies against r-Pfen in mice and rabbit. Here we report our recent attempts to raise specific monoclonal antibodies against the recombinant 6xHis-tagged enolase and a pentadecapeptide encompassing the unique plant like pentapeptide sequence.

Material & Methods

Preparation of r-Pfen: The 6xHis-tagged *P. falciparum* enolase (r-Pfen) was expressed and purified as described earlier[13]. Briefly, the protein was expressed in *E. coli* strain *XL1Blue*. Cultures were transformed with the recombinant plasmid and were grown in Luria-Bertani medium containing 100 μg/ml ampicillin at 37°C. Expression of enolase was induced with IPTG (isopropyl-ß-D-thiogalactopyranoside). The cells were lysed by incubation in 50 mM phosphate-Na^+ (10 ml per g wet weight), pH 8 containing 300 mM NaCl, 1 mg/ml lysozyme, 1 mM PMSF for 30 min on ice and sonicated for six cycles, 15 sec each, with 15 sec cooling between successive bursts at 5 output in Branson Sonifier 450. The lysate was centrifuged at 45,000 g for 30 min in Beckman Ultracentrifuge (Model LE-80K, 70 Ti Rotor). 6xHis-tagged recombinant-Pfen (r-Pfen) was purified from soluble cell extract using Ni-NTA sepharose (Quiagen) affinity chromatography. Soluble cell extract was mixed with Ni-NTA slurry (8 ml per litre culture) for one hour with gentle agitation. Slurry was poured on a column and washed with 50 bed volumes of 50 mM Na-phosphate, 40 mM imidazole, 300 mM NaCl, 1 mM PMSF, 5 mM 2-mercaptoethanol, pH 6 to remove nonspecifically bound proteins. r-Pfen was eluted with 250 mM imidazole in the same buffer. The enzyme was extensively dialysed against phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) (PBS). Purity was assessed by SDS-PAGE and found to be >95% pure. Protein concentration was determined using ε_{280}nm = 41400 $M^{-1}cm^{-1}$.

Cloning and expression of C-terminal & N-terminal fragments of Pfen: C-terminal (aa 165–451, Mw~30 kDa) and N-terminal (aa 1–367, Mw ~40 kDa) fragments of Pfen protein were generated in the following manner. The Pfen has been cloned in *Kpn I* and *Pst I* restriction sites of pQE30 vector. This vector has a *Bam H1* site before *Kpn I* site and there is a *Bam H1* site in the Pfen gene at position 501[13]. The pQE30-Pfen plasmid was digested with *Bam H1* and ethanol precipitated, linearised plasmid was self ligated using T4 DNA ligase. This was used to transform *E. coli* DH5-α and plated on to ampicillin containing LB plates. This resulted in loss of 164 amino acids from N-terminal and a 6xHis tagged C-terminal fragment of 282 amino acids could be obtained (Pfen-N clone). Similarly, pQE30 has a *Hind III* site after the *Pst I* site and also there is a

Hind III site at position 1091 in Pfen. Thus digestion of pQE30-Pfen with *Hind III*, resulted in a linear fragment, which on self ligation produced a construct having 6xHis tagged N-terminal sequence (1–367 aminoacids) of Pfen (Pfen-C clone).

Sequence alignment, 3D structure modeling and peptide property calculation: The enolase peptide sequences were aligned using CLUSTAL W for homology comparisons[14]. The 3D structures of *P. falciparum* enolase was modeled according to the known X-ray structure of yeast enolase (PDB: 1EBG), using the SWISS-MODEL server[15]. Hydrophilicity and net charge of the protein was calculated using peptide property calculator (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp).

Coupling of peptide with bovine serum albumin (BSA): The synthetic peptide -ELDGSKNEWGWSKSK- was obtained from Mimotopes, Clayton South, VIC Australia. The peptide was coupled to BSA using the bifunctional reagent glutaraldehyde. About 2 mg of peptide and 6 mg of BSA were dissolved in 1 ml of PBS. To this 1 ml of 0.4 % gluteraldehyde was added in drop wise fashion along with stirring and allowed to stand for 1 h at room temperature. Subsequently excess of gluteraldehyde was quenched by the addition of 0.5 ml of 1 M glycine in PBS and allowing it to incubate for 1 h. This was extensively dialysed against PBS to remove all small molecules.

Immunisation of mice and establishment of hybridoma: Monoclonal antibodies were generated by the method of Kohler and Milstein[16]. About 50 µg r-Pfen or peptide coupled BSA (P-BSA) was emulsified with Freund's adjuvant and intra-peritoneally administered into six weeks old female BALB/c mice. After four weekly injections, the mice were immunised on a monthly basis for two months. Five days before the fusion of splenocytes with the mouse myeloma Sp2/0 cells, the mouse was immunised once with 250 µg of the immunogen in PBS. Antibody secreting clones were selected by ELISA[17]. For ELISA, polystyrene Maxisorp plate (Nunc, Roskilde, Denmark) was coated with antigen by placing 100 µl of 0.5 mg/ml r-Pfen or P-BSA in each well and allowing it to stand for 6 h at 37°C. Unoccupied sites were blocked with 5% solution of skimmed milk in PBS. Hybridoma culture supernatants (or mAbs) were incubated with the immobilised antigen at 4°C, overnight. Antibodies bound to the antigens were detected by the addition of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Boehringer Mannheim, Germany), followed by addition of 0.5 mg/ml of the substrate ABTS (2,2'-Azino-bis[3-ethylbenziazoline-6-sulfonicacid]) (Boehringer Mannheim, Germany) (in citrate phosphate buffer, pH 4.3) containing 0.03% H_2O_2 and measuring absorbance values at 405 nm in the ELISA reader (EL808 Ultra Microplate reader, Biotek Instruments Inc). ELISA experiments with yeast and rabbit muscle enolases were performed, following the same protocol as described earlier[13]. Finally two mAbs were generated against r-Pfen and six against P-BSA.

Electrophoresis and Western blotting: Proteins were resolved on an SDS/12% polyacrylamide gel[18] and visualised by staining with coomassie brilliant blue R-250. For Western blotting, crude cellular extracts and/or purified r-Pfen were separated on a SDS-gel and were transferred to nitrocellulose membrane using semi-dry Western transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a constant voltage (20 V) for 35 min. The membranes were blocked with 5% skimmed milk in PBS (NaCl/P_i; 137 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 0.05% Tween-20 for 1 h. The blots were treated with the mouse polyclonal or monoclonal antibodies and secondary anti-mouse IgG coupled to horseradish peroxidase. Both antibodies were used at 1: 1000 dilution. The immuno-blots were developed using dianilino-benzene substrate.

For the preparation of *P. falciparum* (strain 3D7) extracts, intracellular parasites from asexual stages were released from infected RBCs by saponin lysis and collected by centrifugation. Parasite pellet was washed with PBS and dissolved in SDS-PAGE sample buffer. The total crude parasite extract was used for Western blot analysis. Typically each lane had extract from $\sim 10^5$ parasites. Protein extracts from human lymphocytes were prepared by collecting blood in anticoagulant EDTA and separating the lymphocytes at 450xg for 15 min at 4°C, on a Ficoll Histopaque density gradient (Amersham Pharmacia Biotech Inc., Piscatway, NJ, USA). The buffy coat was used as the source of human lymphocytes. Crude protein extract was obtained by sonicating the lymphocytes in PBS containing 1 mg/ml each of pepstatin and leupeptin.

Indirect immunofluorescence assay: An immunofluorescence assay was performed on the blood smears obtained from *P. falciparum* human erythrocyte culture. The smears were fixed for 30 sec using chilled methanol and treated with monoclonal antibody at a dilution of 1 : 2 at room temperature for 1 h. This was then stained for 45 min with Alexa Fluor 488-conjugated anti-rabbit IgG. Parasite nuclei were stained with DAPI at a final concentration of 1 μg/ml. The necessary washes were given after each antibody incubation step, and slides were mounted under glass cover slips in 5 μl vectashield mounting medium. Slides were examined using a Nikon fluorescence microscope[19].

Results & Discussion

Monoclonal antibodies against r-Pfen: Although enolases are highly conserved across the species, interesting differences have been noted between apicomplexan parasite enolases and mammalian homologues[20]. One of the main differences is the presence of a pentapeptide sequence ^{104}EWGWS^{108} in *P. falciparum* enolase. A CLUSTAL-W sequence alignment[14] for enolases from several species around this insert is shown in Fig. 1(A). If we model the 3D structure of *P. falciparum* enolase using X-ray structure of yeast enolase (PDB:1EBG) (Fig. 1 B), this pentapeptide insert forms a part of a surface loop structure. It is likely that if this region of parasite

(A)

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Plasmodium      ELDGSKNEWGWSKSK
Toxoplasma      QLDGTKNEWGYSKSK
Arabidopsis     ELDGTQNEWGWCKQK
Human           EMDGTEN-----KSK
RABIT           ELDGTEN-----KSK
YEAST           SLDGTAN-----KSK
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Fig. 1: (A) Partial aminoacid sequence alignment of enolases from several different species. A characteristic plant like pentapeptide insert is present in apicomplexan parasites; & (B) Model structure of *P. falciparum* enolase showing the localisation of this insert and flanking region (^{97}ELDGSKNEWGWSKSK^{111}) in a surface loop

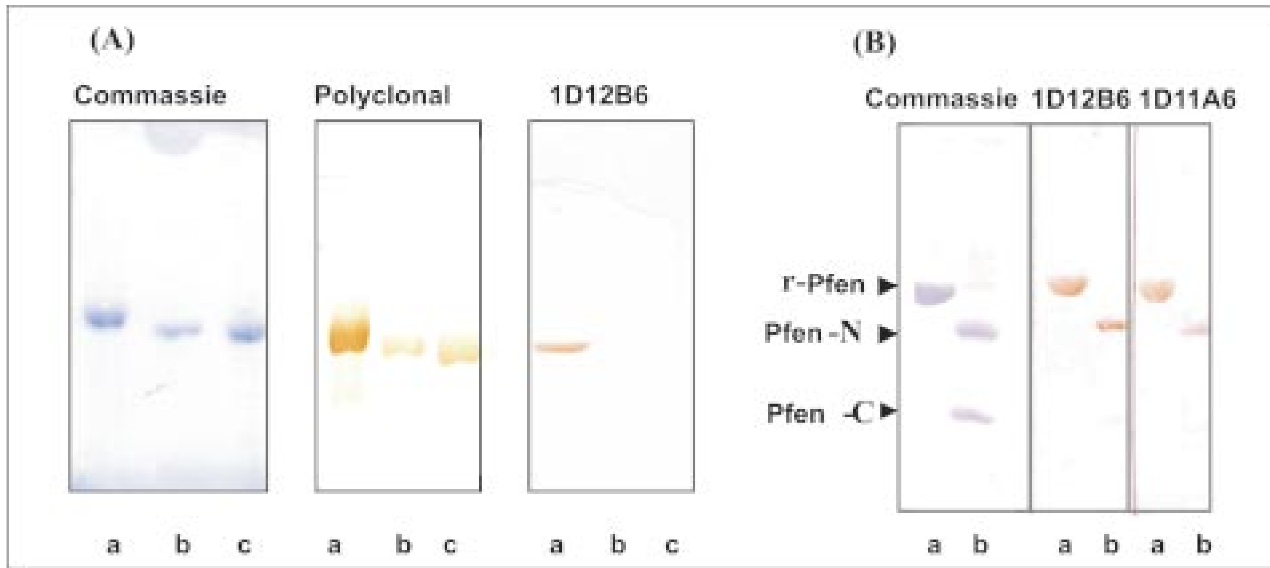

Fig. 2: (A) Comparison of anti-enolase antibodies cross reactivity with (a) r-Pfen, (b) yeast enolase, and (c) rabbit muscle enolase. 10 µg of each enolase protein was run on a SDS-PAGE. First panel shows commassie staining, second panel is a Western blot probed with mouse polyclonal antibody (1:5000) and third panel is probed with monoclonal antibody supernatant 1D12B6 (1 : 10); (B) Mapping of antigenic site(s) of r-Pfen for monoclonal antibodies. Full length r-Pfen (10 µg, MW 51 kDa in lane a) and a mixture of Pfen-N (MW 40 kDa) and Pfen-C (MW 30 kDa) (8 µg each in lane b) were separated on a SDS-PAGE gel. First panel is stained with commassie blue. Second and third panels are Western blots probed with 1D12B6 and 1D11A6 monoclonal antibodies. Note the absence of reactivity with Pfen-C fragment in second and third panel indicating that both these monoclonals are specific for N-terminal sequence of Pfen

enolase is antigenic, we may be able to obtain specific antibodies against this region. For the generation of monoclonal antibodies, mice were challenged with r-Pfen and splenocytes were fused with mouse myeloma cell lines to obtain antibody secreting hybridomas. Initially 10 clones were obtained. However, after few rounds of cell cycle, only two of the clones (D11 and D12) remained as secretors. These parent clones secreted IgG class of antibodies and were sub-cloned to obtain two monoclonals (1D12B6 and 1D11A6). These clones had an antibody titre of 1 : 1000. We also collected the anti-sera from these mice and compared the specificity of polyclonal and monoclonal antibodies against full length r-Pfen. Results are presented in Fig. 2(A). Equal amounts (10 µg) of r-Pfen, yeast and rabbit muscle enolases were run on a 12% SDS-PAGE and blotted on nitrocellulose membranes. These were then probed with either polyclonal sera or with monoclonal (1D12B6) antibodies. Polyclonal antibodies exhibit lot more reactivity with r-Pfen as compared to yeast and rabbit proteins at a dilution of 1 : 5000. Although these proteins are highly homologous (61–68%), we observed considerably high specificity against r-Pfen. However, these polyclonal antibodies were not completely exclusive for r-Pfen, and cross reacted with the yeast and rabbit enolases (Fig. 2). In comparison monoclonal antibody 1D12B6 (1:10), exclusive reactivity to r-Pfen was observed (Fig. 2).

In order to map the antigenic region(s) for the monoclonal antibodies, we generated two deletion clones of r-Pfen covering N-terminal (Pfen-N, aa 1–367) and C-terminal (Pfen-C, aa 165–451) as 6xHis tagged proteins, expressed in *Escherichia coli* and purified by Ni-NTA chromatography. These deletion proteins were used to map the location of specific

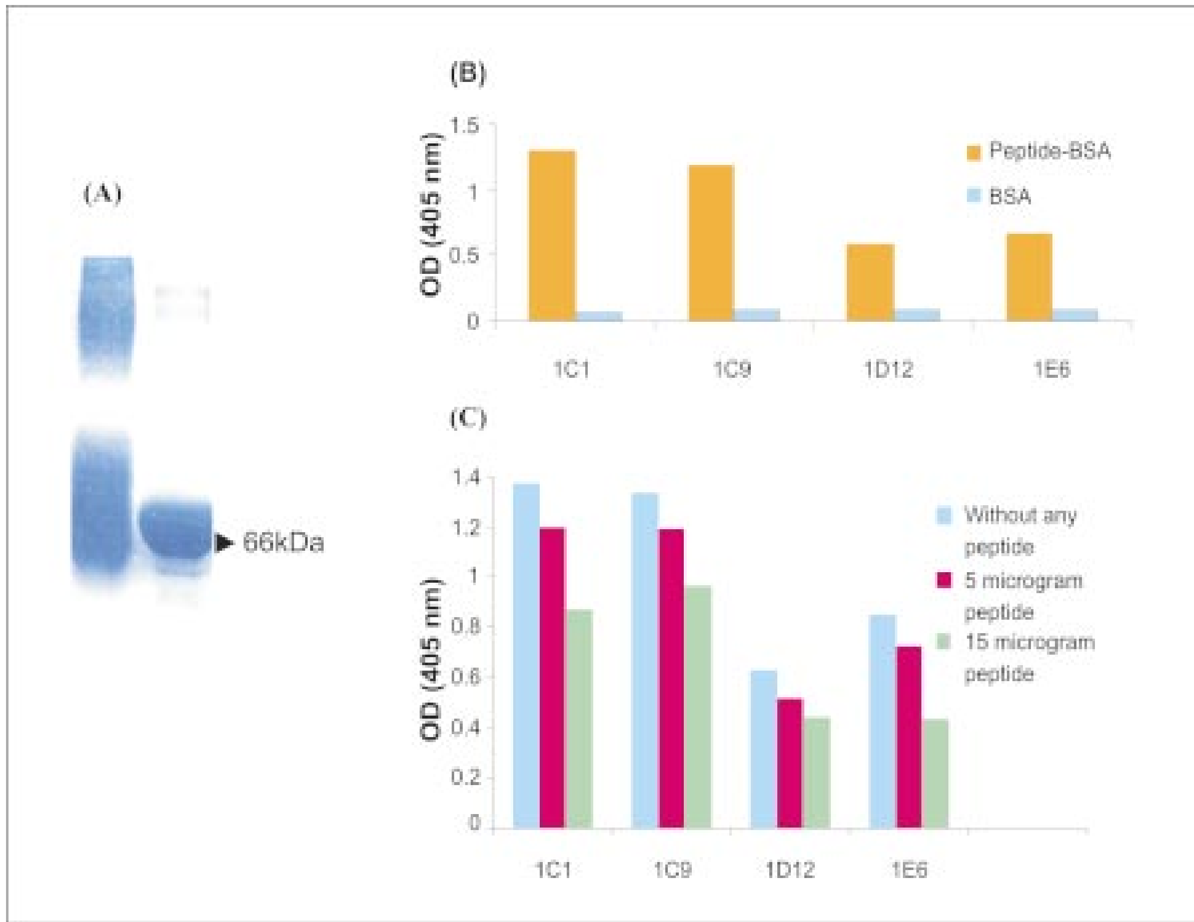

Fig. 3: (A) SDS-PAGE analysis of peptide coupled BSA (P-BSA) (lane 1). Lane 2 is pure BSA. Chemical cross linking of peptide also cross links BSA dimers (lane 1, two smears); (B) Reactivity of parent peptide clone supernatants (1C1, 1C9, 1D12 and 1E6) against P-BSA and BSA as measured by ELISA. Plates were coated with 500 ng of P-BSA or BSA and probed with different supernatant antibodies; and (C) Competitive displacement of antibodies by the synthetic peptide. Supernatant antibodies were incubated with 5 or 15 µg of peptide for 30 min and then used to probe the ELISA plates coated with 500 ng of P-BSA

antigenic epitopes against which the monoclonals are directed. r-Pfen and a mixture of Pfen-C and Pfen-N (8 µg each) were separated on a 12% SDS-PAGE and probed with two monoclonal antibodies. Results are presented in Fig. 2(B). Commassie stained panel shows the presence of full length and deletion fragments of Pfen at appropriate sizes. In the Western blot, we observed that both monoclonal antibodies bind to epitopes located on the Pfen-N fragment. Absence of cross reactivity with Pfen-C would suggest that the antigenic site is located in the first 165 amino acids of r-Pfen. It is interesting to note that the unique plant like pentapeptide insert (-^{104}EWGWS^{108}-) is a part of this sequence.

Antibodies against peptide (ELDGSKNEWGWS-KSK) coupled bovine serum albumin: In order to generate a specific antibody directed against the unique pentapeptide sequence in Pfen, we examined the properties of the flanking sequences on either side of this insert. We selected the region which includes maximal number of lysines in the sequence to make it hydrophilic, soluble and antigenic. A sequence of fifteen amino acids,

^{97}ELDGSKNEWGWSKSK^{111} was chosen. This sequence had an average hydrophilicity value of 0.7 on Hopp & Woods scale[21] and is likely to be antigenic. In general, it is believed that if a molecule is highly charged, it may be difficult to create the complementary antibody combining site with charge groups. The selected peptide has a good balance of electronegative and electropositive residues so as to bear zero net charge at pH ~7. Thus at physiological pH, it is not likely to have any net charge. As small molecules are known to be feeble immunogens and evoke very weak immune response, we coupled this peptide to bovine serum albumin using gluter-aldehyde. On SDS-PAGE this coupled conjugate gave two smears which are likely to be due to coupling of the peptide with monomeric and dimeric forms of the BSA (P-BSA) (Fig. 3A). Peptide conjugated-BSA was used to immunise the mice and splenocytes from these mice were used to generate hybridomas. This resulted in the isolation of four secreting parent clones. The antibodies secreted by all these clones were of IgM type. Specificity of these monoclonal antibodies for BSA and P-BSA was examined by ELISA. Results are presented in Fig. 3 (B). These monoclonals had very poor cross reactivity to BSA as compared to P-BSA. To further confirm that antibodies are peptide specific, we performed a peptide competitive displacement experiment. ELISA was performed in the presence of 5 and 15 μg of peptide. The ELISA values decreased with respect to the control for all four clones (Fig. 3C), suggesting that indeed these antibodies are directed against the peptide.

It is a common observation that antibodies raised against peptides are usually not the native protein reactive antibodies (NPRA). Such situations arise when either the antigenic epitope is buried or inacce-ssible in native protein or the segment containing the peptide sequence in native protein is too rigid to allow the anti-peptide antibody to 'mold' it to fit the binding site. We examined the question whether the anti-peptide antibodies bind to the native protein by measuring the cross reactivity of the antibodies against r-Pfen (native full length enolase) using ELISA. Fig. 4(A) shows the reactivity of two of the parent clones (1D12 and 1C9) against r-Pfen and P-BSA. Both antibodies were able to interact with the

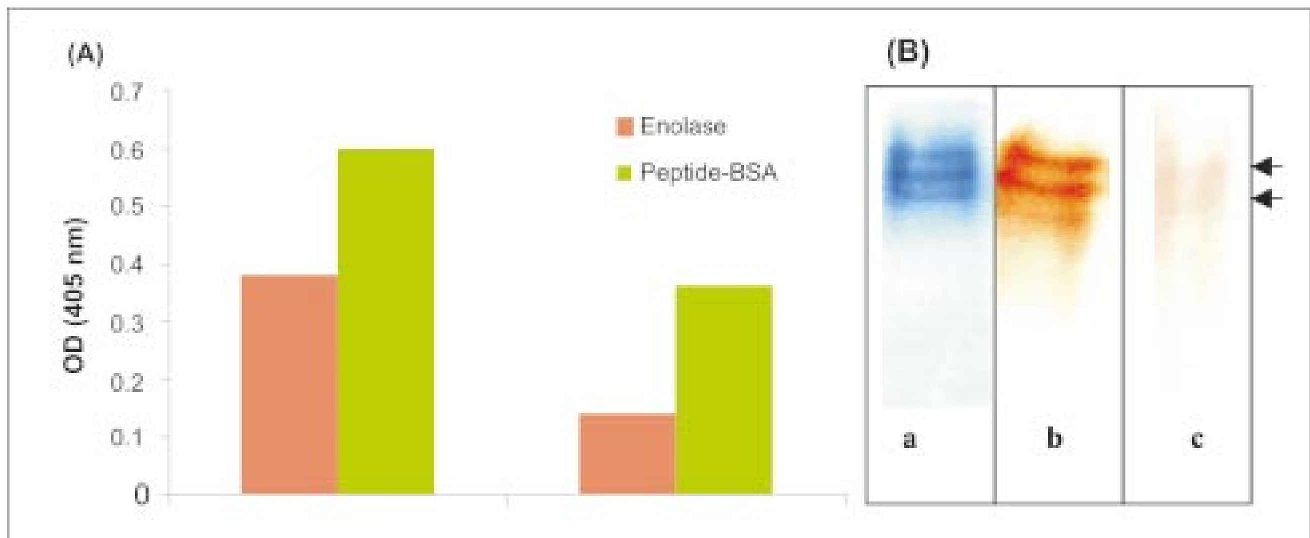

Fig. 4: (A) Reactivity of anti-peptide antibody parent clone supernatants against native enolase as measured by ELISA. Plates were coated with 500 ng of enolase (r-Pfen) or P-BSA; & (B) Western blot analysis for reactivity of native enolase with monoclonal antibody 1C9G12. 10 μg of r-Pfen was resolved on 8% native PAGE and (a) visualised by commassie staining, (b) blot probed with anti-r-Pfen polyclonal antibody (1 : 5000), and (c) blot probed with 1C9G12 monoclonal antibody (1 : 20)

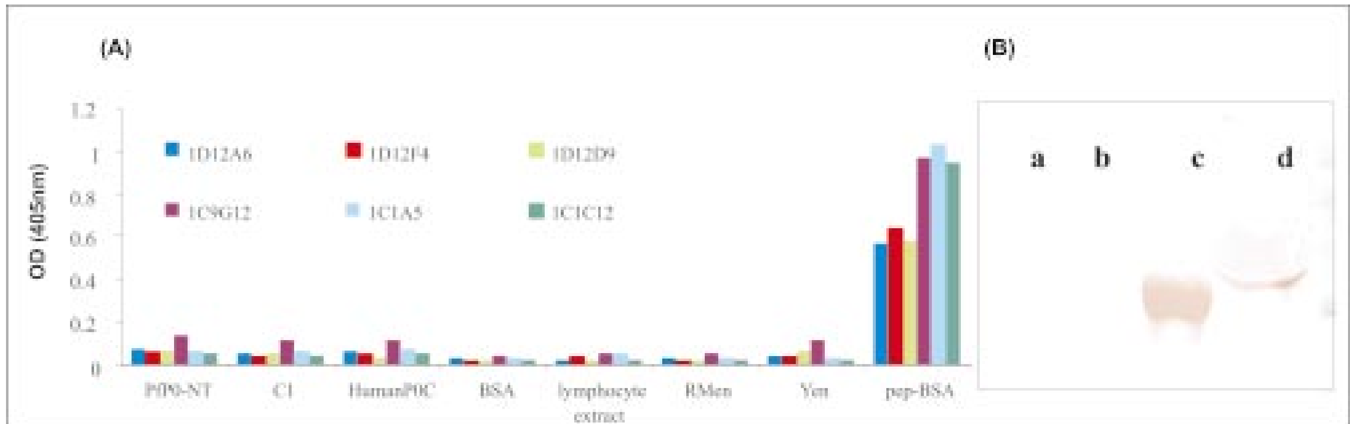

Fig. 5: Specificity of different monoclonal antibodies raised against P-BSA: (A) comparison of ELISA reactivity of monoclonal antibodies against 500 ng of different antigens (a), (b) and (c) are NT, C1 and C0 regions of ribosomal PfP0 protein of *P. falciparum*[22], (d) BSA, (e) human lymphocyte extract, (f) rabbit muscle enolase, (g) yeast enolase, and (h) P-BSA; and (B) Comparison of immuno-blot reactivity against (a) rabbit muscle enolase, (b) yeast enolase, (c) r-Pfen; and (d) *P. falciparum* extract, run on 12% SDS-PAGE gel and probed with 1C9G12 monoclonal antibody (1 : 20)

Fig. 6: Immunofluorescence assay with *P. falciparum* infected human red blood cells treated with (A) DAPI (1 μg ml^{-1}); (B) 1C9G12 (1 : 2) monoclonal antibody; and (C) bright field picture. Two different fields are shown. Three horizontal panels represent the same field. Uninfected erythrocytes are marked by arrows

native enolase.

mAb 1C9G12 was tested on a blot of a native gel to check whether it could interact with various oligomeric forms of the enolase. In Fig. 4(B), panel (a) shows a commassie stain of r-Pfen in a native gel. Two major bands are observed and both of these bands cross react with polyclonal (panel b) as well as monoclonal antibody (panel c). Cross reactivity with the native enolase protein supports the conclusion that the antigenic peptide sequence is located on the surface in the 3D native structure of Pfen. The modeled structure of Pfen also predicts that this pentapeptide containing sequence forms a surface loop in the folded protein (Fig. 1B).

Six different anti-peptide monoclonal antibodies were then obtained from three different parent clones—1D2A6, 1D12F4, 1D12D9, 1C9G12, 1C1A5 and 1C1C12. Of these, the six anti P-BSA mAbs were assayed for specificity against several different antigens. Antigens used were N-terminal and C-terminal fragments of *P. falciparum* ribosomal protein P0 (PfP0-NT, C1 region of PfP0), C-terminal fragment of human P0, bovine serum albumin, human lymphocyte extract, enolases from rabbit and yeast and P-BSA. Results are presented in Fig. 5(A). The high reactivity of all monoclonals with P-BSA and negligible cross reactivity with other antigens, reiterates our earlier conclusion that these monoclonals are highly specific for r-Pfen derived peptide. High selectivity in cross reactivity of monoclonal 1C9G12 is further evident from the immunoblot of a 12% SDS-PAGE gel, where rabbit muscle and yeast enolases fail to cross react, whereas r-Pfen and enolase from parasite extract gets unequivocally identified (Fig. 5B).

We also examined the ability of 1C9G12 monoclonal antibody to interact with *P. falciparum* in erythrocyte cultures. In an immuno-fluorescence assay (IFA), the parasite infected RBC's could be visualised by staining with DAPI and same cells could also be stained with anti-peptide antibody 1C9G12 (Fig. 6). As the parasite culture used here is not synchronised, several different stages of parasite are visible and all of these stages are stained with the antibody. This confirms our finding from homology modeling that this epitope is exposed on the surface of the molecule. It is important to note that all the anti-peptide monoclonal antibodies obtained are of IgM class.

Conclusion

We have raised monoclonal antibodies against *P. falciparum* enolase using two different antigens, (i) with native recombinant enolase protein (r-Pfen), and (ii) with a unique pentapeptide sequence containing peptide coupled to BSA (P-BSA). Two monoclonals (1D12B6 and 1D11A6) obtained with r-Pfen as antigen, are of IgG class and could be mapped to bind to antigenic site(s) located in the N-terminal region (aa 1–165) of the Pfen. Six monoclonals obtained against the P-BSA were of IgM type and are able to bind to the native enolase as confirmed by ELISA, Western blotting and immunofluorescence assay on parasite culture. All these monoclonals are very specific for *P. falciparum* enolase and do not cross react to rabbit muscle or yeast enolases. Since this peptide encompasses the unique pentapeptide insert which is a characteristic feature of enolases from apicomplexan parasites, the observed cross reactivity with native Pfen can be taken as evidence for the surface localisation of this peptide sequence in Pfen native structure. The high specificity of these monoclonals against Pfen makes them excellent reagents for parasite detection in infected blood samples.

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