Recombinant antibodies specific for the Plasmodium falciparum histidine-rich protein 2

Elisabeth Ravaoarisoa,1,2 Halima Zamanka,2,3 Thierry Fusai,4 Jacques Bellalou,5 Hugues Bedouelle,6 Odile Mercereau-Puijalon2 and Thierry Fandeur2,3,*

1Institut Pasteur de Madagascar; Unité de recherche sur le Paludisme; Madagascar; 2Institut Pasteur; Immunologie moléculaire des parasites; CNRS URA 2581; France; 3Centre de Recherche Médicale et Sanitaire; Unité de Parasitologie; Niger; 4Institut de Médecine Tropicale du Service de Santé des Armées; Unité de Recherche en Biologie et Epidémiologie Parasitaire; France; 5Institut Pasteur; Module de Protéines Recombinantes; Plate-forme 5; France; 6Institut Pasteur; Unité de Prévention et Thérapie Moléculaires des Maladies Humaines; CNRS URA3012; France

Key words: Plasmodium falciparum, malaria, histidine-rich protein, monoclonal antibodies, recombinant Fab, rapid diagnostic test

Early diagnosis and appropriate treatment are key elements of malaria control programs in endemic areas. A major step forward in recent years has been the production and use of rapid diagnostic tests (RDTs) in settings where microscopy is impracticable. Many current RDTs target the Plasmodium falciparum histidine-rich protein 2 (PfHRP2) released in the plasma of infected individuals. These RDTs have had an indisputably positive effect on malaria management, but still present several limitations, including the poor characterization of the commercial monoclonal antibodies (mAbs) used for PfHRP2 detection, variable sensitivity and specificity and high costs. RDT use is further limited by impaired stability caused by temperature fluctuations during transport and uncontrolled storage in field-based facilities. To circumvent such drawbacks, an alternative could be the development of well-characterized, stabilized recombinant antibodies, with high binding affinity and specificity. Here, we report the characterization of the cDNA sequences encoding the Fab fragment of F1110 and F1546, two novel anti-PfHRP2 mAbs. FabF1546 was produced in the Escherichia coli periplasm. Its properties of binding to the parasite and to a recombinant PfHRP-2 antigen were similar to those of the parental mAb. As the affinity and stability of recombinant antibodies can be improved by protein engineering, our results open a novel approach for the development of an improved RDT for malaria diagnosis.

Introduction

Plasmodium falciparum malaria remains one of the leading causes of morbidity and mortality in tropical areas. Early diagnosis is a key element of malaria control programs, as it allows prompt and appropriate treatment of clinical malaria, reducing the risk of progression to severe disease. In addition, the artemisinin-based combination therapies are expensive and their high cost increases the need for simple and accurate parasite-based diagnosis for malaria. Microscopic diagnosis of blood specimens is sensitive and specific, but difficult to apply in the field because of the need for specific equipment and experienced technical staff that are rarely available at the community level and time-consuming slide inspection for accurate quantification and species determination. Alternative immunodiagnostic approaches that are suitable for use in field conditions have been developed. A major advance in the recent years has been the deployment of rapid diagnostic tests (RDT) in settings in which microscopy is not possible.

Most of the currently available RDTs for malaria are based on detection of the P. falciparum histidine-rich protein 2 (PfHRP2) by monoclonal antibodies (mAbs). The PfHRP2 protein contains central repeats, rich in alanine and histidine residues, the number of which varies between parasite clones. This abundant protein is soluble and heat-stable, produced specifically by P. falciparum, and absent from other malaria parasites infecting humans. It is an interesting and sensitive target antigen for detecting this species in biological fluids. Several companies manufacture PfHRP2-based RDTs, the performances of which have recently been assessed and compared. Comparative data from a large panel of malaria RDTs against P. falciparum samples adjusted at low and high parasite densities showed that only about one third (13/33) of commercial tests have a good sensitivity at low parasite density (200 parasites/µL of blood). As a
and are much less sensitive in conditions of lower parasitemia. Sensitivity of RDTs is adversely affected by temperature fluctuation during transport and uncontrolled storage in field facilities. Sensitivity when parasite density exceeds 100 parasites/μL and are much less sensitive in conditions of lower parasitemia.

PfHRP2-based RDTs have had an indisputably positive effect on malaria management in terms of patient care, but they remain subject to several limitations. Their cost, which reaches that of an artemisinin-based combination therapy, is unaffordable for most populations at risk. The RDTs produced by different manufacturers differ in terms of sensitivity and specificity when parasite density exceeds 100 parasites/μL and are much less sensitive in conditions of lower parasitemia.

Results

Characterization of novel anti-PfHRP2 monoclonal antibodies. Hybridoma cell lines were constructed from mice that had been immunized with P. falciparum asexual blood stages. We screened the secreted antibodies for their reactivity towards PfHRP2 by western blotting and indirect immunofluorescence experiments. A representative western blot is shown in Figure 1 (left) for two of the selected mAbs, mAbF1110 and mAbF1546, which belonged to the IgGk isotype. As reported previously for mAbs reacting with PfHRP2, a multiple banding pattern, ranging from 50–35 kDa, was observed with mAbF1110 and mAbF1546. These antibodies recognized predominantly 50 and 37 kDa protein species, corresponding to the PfHRP2 and PfHRP3 polypeptides, respectively. They also reacted with a 33 kDa species, whose intensity varied depending on the particular antigen extract. We assumed that it corresponded to a proteolysis product of larger polypeptides, possibly PfHRP2 or PfHRP3. However, we cannot rule out the possibility that this species resulted from cross-reaction with another as yet unidentified parasite histidine-rich polypeptide.

Air-dried erythrocytes infected with P. falciparum late stages showed a typical coarse fragmented or dotted pattern of fluorescence in indirect immunofluorescence microscopy, whereas the ring forms of the parasite showed a weaker and more diffuse fluorescence (Fig. 1, right). These patterns are consistent with antibody reactivity to PfHRP2. The FITC conjugate alone or supernatants from non-fused melanoma cells did not produce any significant positive signal on air-dried parasites at similar dilutions. This absence of signal indicated that the tested antibodies were specific for infected red blood cells (IRBC). Air dried fixation was preferred over acetone treatment as the former is known to better preserve the integrity of parasite proteins while keeping them mostly in a native form. The specificity of mAbF1110 and mAbF1546 for PfHRP2 was confirmed by using a recombinant PfHRP2 protein, fused with the maltose binding protein (see below).

To verify the sensitivity and linearity of HRP-2 detection by the selected mAbs, antigenic extracts containing known amounts of in vitro-cultured P. falciparum-parasitized erythrocytes were prepared and examined by sandwich ELISA using mAbF1110 or mAbF1546 mAbs as capture antibodies, biotinylated mAbF1546 as the primary antibody, and an avidin-peroxydase conjugate as a detection reagent. The mean reactivity for duplicate measures of parasite densities equivalent to 500–0.25 parasite per μl are shown in Figure 2. Typical positive dose response curves of the cDNAs encoding their Fab fragments, their nucleotide sequence and the expression and characterization of the recombinant Fab. The ability of a recombinant Fab fragment to bind native and recombinant PfHRP2 compared well with the parental mAb. Our results provide the first molecular characterization of antibodies with specificity for PfHRP2 and a solid basis for improving their performance and stability for applications in malaria diagnosis.

Figure 1. Reactivity of mAbF1546 and mAbF1110 with P. falciparum as assayed by western blots and indirect immunofluorescence. The western blot assayed the reactivity of mAbF1546 (lane 1) and mAbF1110 (lane 2) with P. falciparum crude antigenic extracts, subjected to electrophoresis under denaturing conditions. Apparent MW (kDa) are shown on the left side of the immunoblot. The indirect immunofluorescence staining patterns for mAbF1546 (part 1) and mAbF1110 (part 2) were performed on air-dried blood stages of P. falciparum. Antigenic preparations were obtained from 3D7 parasites.
or heterologous mAbF1110 (Fig. 2B) was used for immunocapture. In all cases, the assay was found suitable for the detection of PfHRP2, freed from IRBC by hypotonic shock or mild denaturation with 0.05% SDS. Measures were slightly higher after treatment with 0.05% SDS. This observation indicated that SDS at low concentration might help PfHRP2 recovery without affecting the recognition by mAbs. The detection limit of this assay was comprised between one to four parasites/µl with a linear range of 4–125 parasites/µl. The fact that mAb (F1546) can be used for both capture and detection in a single assay, indicated that the targeted epitope was likely repeated and easily accessible. MAbF1546 could therefore be used either in combination with F1110 or alone for antigen immunocapture and detection without significant decrease in sensitivity. This may have interesting application for diagnosis purpose.

Sequence analysis. The VL-CL and VH-CH1 antibody gene fragments, derived from mouse monoclonal hybridoma cell lines F1110 and F1546, were generated by amplification of the corresponding cDNAs by PCR, using a set of degenerate primers. The CKDNA-3’ and LC7-5’ primers amplified a 678 bp PCR product from both F1546 and F1110 cDNAs, corresponding to their VL-CL domain. The VH-CH1 fragments were produced at high yield with a single 3’-primer, ClgG1, and two different 5’-primers, VHI (for F1546) and VHIIb (for F1110), producing 657 and 654 bp fragments from the F1546 and F1110 cDNAs, respectively. The various VL-CL and VH-CH1 amplification fragments were inserted into the pGEM-T vector. Three to five independent clones were sequenced and aligned for each molecular species making it possible to generate a consensus sequence for each chain. Pair-wise alignment of the F1546 and F1110 VL-CL and VH-CH1 consensus protein sequences showed that the monoclonal hybridoma cell lines F1546 and F1110 used the same CL-VL, but that their CH1-VH1 sequences differed, particularly in the variable regions, which displayed only 38% identity at protein level (Fig. 3). Thus, despite their similar pattern of reactivity on immunoblotting and immunofluorescence and their identical CL-VL germline, the monoclonal hybridoma cell lines F1110 and F1546 are different.

Computational analysis of the VH and VL sequences. The VH and VL domain sequences were analyzed with IMGT V-Quest software which identifies the immunoglobulin germline V, D and J genes from which a specific immunoglobulin chain is derived. Sequence analysis of the VL fragment shared by hybridoma cell lines F1546 and F1110 confirmed that it belonged to the mouse K chain subgroup. Maximum sequence identities of 96.2% (280/291 nt) and 97.3% (36/37 nt) were observed with the V gene IGKV3-5*01 [K02161] and IGKJ1*1 [V00777] alleles, respectively (Fig. 4A). Fourteen base pair differences from the germline sequence were observed in the \( \text{IGHV1S22}^*01 \) allele, with respect to the closest germline sequence IGHV1S22^*01, were observed in the

![Figure 2. Reactivity of mAbF1546 and sandwich ELISA with mAbF1546 and mAbF1110.](image-url)
F1110-VH domain, resulting in eight amino-acid substitutions: Q5E, S9A and K20M in FR1; T65A in CDR2; G76A, M89L and D98G in FR3; and R106N in CDR3 (Fig. 4B). The F1546-VH sequence was the closest to the IGHV3-8*02 [AJ972403] allele, with which it displayed 97.5% (269/276 nt) identity. Eleven base pair differences were detected in F1546-VH fragment compared to the IGHV3-8*02 germline sequence, leading to seven amino-acid substitutions: Q5E in FR1; Y52F and Y55S in FR2; S74G, K84N and Q86H in FR3; and R106N in CDR3 (Fig. 4C). Amino-acid substitutions at positions Q5E and R106N were observed in both VH sequences, suggesting that a glutamine residue in position 5 and an asparagines residue in position 106 in CDR3 may be essential for antibody stability or antigen recognition. The CDRs of antibodies, and CDR3 in particular, are generally responsible for high-affinity binding. The J and D regions of both mAbs were the most similar to the IGJH2*01 [V000770] and IGHD4-1*01 alleles, respectively.

**Fab expression.** The pF1546 and pF1110 plasmids harboring the assembled VL-CL and VH1-CH1 fragments cloned into the pPE1 vector were used to transform the E. coli HB2151 strain. FabF1546-H6 and FabF1110-H6 antibody fragment have a C-terminal hexahistidine tag. After mild IPTG induction, a soluble recombinant Fab fragment was harvested from periplasmic extracts and purified. The purified fractions were analyzed by SDS-PAGE under reducing or non-reducing conditions followed by immunoblotting. The crude periplasmic extracts gave a complex pattern of bands in the lower part of the gel, with two major bands in the 48 and 23 kDa regions (Fig. 5, lanes 1 and 2). Chromatography-purified Fab fragments migrated as a single band in the 48 kDa region of the gel under non reducing conditions, corresponding to intact recombinant Fab, and as a 26 kDa band after reduction, corresponding to the VL-CL and VH1-CH1 fragments (lanes 3 and 4, and lanes 5 and 6, respectively). Somewhat larger yields were obtained for FabF1546-H6 fragment. We therefore selected the FabF1546-H6 fragment for further studies of binding properties.

**Recombinant MalE-PfHRP2.** The MalE-PfHRP2 hybrid protein was produced in a soluble state and at high yield by the induced recombinant E. coli strain HB2151 (pER1). The MalE-PfHRP2 was recognized by human sera from endemic areas and by all commercial RDTs targeting PfHRP2 that we tested (data not shown). An example of such a reactivity is shown in Figure 6 for the reagents provided in the CareStart Malaria Combo test (Access Bio-Inc.). The performance of the CareStart device, which is based on both HRP2 (Pf specific) and pLDH (pan specific) detections, has been evaluated recently in comparison with other commercial malaria RDT and ranked amongst the best.18 The soluble extract from induced HB2151(pER1) cells reacted strongly with P. falciparum test zone 1 of the device, based on PfHRP2 recognition, and with the control zone C, whereas the panspecific test zone 2, based on pLDH, did not react, as expected (lane 2). This pattern is similar to the pattern of P. falciparum-infected blood samples, demonstrating that the RDT reactivity of the recombinant MalE-PfHRP2 protein is similar to the native PfHRP2 produced by wild P. falciparum.
the parasite extract and gave a signal above background (Fig. 8A).

The binding specificity of FabF1546-H6 was similarly analyzed by direct ELISA, using purified recombinant MalE-PfHRP2 at concentrations ranging from 20 μg.mL^{-1} to 1.5 ng.mL^{-1}. A concentration-dependent response was again clearly observed, with reactivity above the technical threshold at MalE-PfHRP2 concentrations down to 20 ng.mL^{-1} (Fig. 8B). This sensitivity was in the same range as that previously reported for an HRP2-specific ELISA assay based on a recombinant HRP2 and two mAbs, 1E1 and 2G12.15,31 We observed that the recombinant antibody fragment had a lower reactivity with native and recombinant PfHRP2 than the parental mAb. This difference in reactivity comes probably from the fact that IgGs are bivalent and Fabs monovalent. Moreover, we used alkaline phosphatase conjugates, which have lower catalytic turnovers than horseradish peroxidase conjugates. Overall, our results indicated that the pattern of PfHRP2 recognition by FabF1546-H6 and the parental mAb were essentially identical.

Discussion

Several millions of malaria RDTs, mostly specific for *P. falciparum*, are purchased every year for malaria control programs in endemic countries, but the world consumption of commercial RDTs will continue to increase in the coming years because of the recently published WHO guidelines recommend...
a parasitological confirmation of diagnosis in all patients suspected of having malaria before treatment. Implementation of this recommendation will require mobilization of important financial and human resources. The advent of RDTs has made such testing possible when microscopy would be difficult or impossible. Many current RDTs are based on the detection of *P. falciparum* HRP2, a soluble parasite antigen specific to *P. falciparum* that is considered the primary immunological target for *P. falciparum* malaria testing. A large body of information from field trials that assessed the impact on RDT specificity and sensitivity of parameters such as manufacturer, parasite polymorphism and stability to heat, or comparing the performance of RDTs with conventional methods such as microscopy has recently accumulated. Paradoxically, little information about the *Pf* HRP2 antibodies used in these commercial tests has been published. For example, the isotype (IgG or IgM), subclass, epitope targeted (unique or repeated), molecular structure and laboratory origin of these antibodies remain essentially unknown. All the *Pf* HRP2 antibodies in current use were developed at the end of the 1980s and most commercial RDTs are probably manufactured using reagents purchased from few suppliers producing the mAbs on a large scale. Given the increasing demand for RDTs, a better characterization of antibodies used for malaria diagnosis and distribution of this information to RDT users are required.

In this study, we produced and characterized two novel mAbs against *Pf* HRP2. In indirect immunofluorescence studies, these antibodies reacted over the entire infected cell, producing a granular pattern of staining typical of antibodies reacting with *Pf* HRP2. In immunoblot experiments, both mAbs identified a multiplet of proteins, with two major bands at 37 kDa and 50 kDa. An additional band was also detected at about 35 kDa. The identification of multiple bands is consistent with previous reports on mAbs reacting with *Pf* HRP2. In ELISA, the F1110 and F1546 antibodies could be used either alone or together, for antigen immunocapture and detection with no significant loss of sensitivity. The targeted epitopes are therefore repeated and possibly different. They are likely linear because recognition by these antibodies was not affected by SDS or treatment with reducing agents. A study evaluating series of five peptides, derived from the central part of the *Pf* HRP2 molecule, showed that only antibodies raised against the CGDHHAADAHATDAHHGC peptide cross-reacted with *Pf* HRP3. Additional studies showed that most anti-HRP2 mAbs recognized DAHHAHHA as the major epitope, with possible substitutions replacing the first and last amino acids by Y or V. We anticipate that the F1110 and F1546 antibodies react with closely related epitopes, but careful mapping is required to confirm this hypothesis.

Commercially available tests are sensitive and specific, but there is still a need to establish simple and reliable procedures for ensuring that malaria RDTs meet high criteria of quality before their distribution in the field. Surprisingly, only the enzymatic test is performed on nitrocellulose strips (capture of labeled antibodies by bound antibody). There is no control of mAb reactivity with the targeted antigen. Quality control procedures carried out by WHO at reference laboratories in areas of endemic malaria, are based on the use of calibrated parasite samples obtained from patients. However, patient parasites are polymorphic and therefore the sequence of *Pf* HRP2 and possibly its expression levels may vary between parasite clones. These variations may bias the correlation between parasite number and HRP2 amount. The affinity-purified recombinant Fab-H6 were also subjected to electrophoresis under non-reducing conditions (lanes 1 and 2). The affinity-purified recombinant Fab-H6 were also subjected to electrophoresis under non-reducing conditions (lanes 3 and 4) or reducing conditions (lanes 5 and 6). Lanes 1, 3 and 5, FabF1546-H6; lanes 2, 4 and 6, FabF1110-H6.
diagnostic applications, can therefore be obtained with careful optimization of the production conditions. It is also possible to refine the design of the fragment without altering the affinity for its antigen. The constant or variable domains can be engineered to improve the labeling or affinity properties of the antibody fragment for sensitive immunoassay applications, especially in detecting asymptomatic carriers who represent an important reservoir from which parasite infections may spread and interfere with the global malaria eradication program. Finally, the stability of recombinant antibody fragments can be improved by designing or selecting mutations conferring resistance to denaturation. Improvements in stability should make RDT devices more robust to heat and significantly increase their shelf-life. Fab fragments are generally considered to be more stable than scFv due to the presence of two disulfide bonds. Several approaches have been described for the prediction of stabilizing mutations in recombinant antibody fragments. These methods are based on the observation that the effects of the mutations are additive, making it possible to adjust their combinations and achieve optimal stability.

We described here the first steps on the path towards the engineering of antibodies reacting with \( \text{PfHRP2} \) by recombinant techniques. We identified cDNAs encoding the variable domains of two mAbs, F1546 and F1110, directed against \( \text{PfHRP2} \). These Fab fragments have the same VL but different heavy-chain VH-CDR structures, suggesting that they react with different epitopes. Both Fab fragments were produced in good yields in the periplasm of \( \text{E. coli} \). Yields were particularly high for FabF1546-H6, which was further characterized here. The recombinant FabF1546-H6 displayed a binding specificity for the parasite and recombinant HRP2 proteins similar to that of the parental mAb. The proteins were recognized in a concentration-dependent manner by ELISA and produced similar banding patterns on western blots, probed with either the parental mAb or with the recombinant Fab fragment.

Figure 6. Reactivity of the recombinant MalE-\( \text{PfHRP2} \) protein in the commercial CareStart Malaria Combo test. Crude soluble fraction prepared from non-transfected HB2151 control cells (lane 1) and crude soluble fraction from HB2151 (pER1) cells expressing MalE-\( \text{PfHRP2} \) grown with IPTG (lane 2).

In conclusion, the mAbs and Fab fragments directed against \( \text{PfHRP2} \) described here offer attractive perspectives for the development of improved rapid diagnostic tests for malaria. Inclusion of the recombinant \( \text{PfHRP2} \) antigen would provide the added value of a positive antigen/antibody reaction control and even open the possibility of designing a quantitative assay of the amount of antigen present in the sample rather than a presence/absence testing. Although further studies are required to assess the potential of our results for use in diagnostic applications, they constitute an important step towards the development of a novel generation of diagnostic tests for malaria.

**Materials and Methods**

**Parasites.** The 3D7 and the Palo Alto Marburg (PAM) strains of \( \text{P. falciparum} \) were used in this work. Parasites were maintained in asynchronous cultures in human blood group O+ red blood cells in RPMI 1640 supplemented with 10% AB human serum, 2 g.L\(^{-1}\) glucose, 20 mg.L\(^{-1}\) hypoxanthine, 9.1 g.L\(^{-1}\) hepes 1 M, 2 g.L\(^{-1}\) NaHCO\(_3\) and 2.5 mg.L\(^{-1}\) gentamicin, essentially as described by Trager and Jensen. These strains regularly produce
high parasitemias in vitro and express the P/HRP2 and P/HRP3 antigens.

Monoclonal antibodies. MAb s used in this study were produced in BALB/c mice immunized with schizonts of P. falciparum as described elsewhere. The hybridomas were initially screened for reactivity with P. falciparum on air-dried parasites by indirect immuno-fluorescence. The F1110 and F1546 MAb s were further selected on the basis of their reactivity for P/HRP2 using recombinant antigen. Culture supernatants or ascitic fluids from BALB/c mice bearing the hybridomas were used as sources of MAb s as indicated in the legend of the figures. Isotyping of mouse immunoglobulins was performed using culture supernatant on Isostrips™ mouse MAb isotyping Kit (Santa Cruz Biotechnology) according to manufacturer recommendations.

Immunofluorescence assay. The immunofluorescence assay was performed using air-dried 3D7 parasites as follows. Smears of infected red blood cells (IRBC) adjusted to 1% parasitemia with PBS were made on multi-spot microscope slides and stored at -80°C until use. Air-dried spots where thawed and reacted with a 1:10 dilution of culture supernatant of MAb F1546 and F1110 then incubated for 30 min. at 37°C. Slides were washed 3 times with PBS and stained for additional 30 min. at 37°C with a 1:50 dilution of a fluorescein-conjugate goat anti-mouse IgG (F9006 Sigma). The slides were mounted in PBS containing 10% glycerol and examined at X600 with a fluorescence microscope.

Sandwich ELISA assay for the reactivity of F1110 and F1546 MAb s. Microtiter plates (Maxisorp, Nunc) were coated overnight at 4°C with 100 μl/well of the capture antibody F1110 or F1546 adjusted at 10 μg.mL⁻¹ in PBS. The plates were washed and saturated with 200 μl/well of 1% BSA in PBS-0.05% Tween20 (PBS-T) for 1 h and 100 μl of decreasing concentrations of a soluble antigenic fraction were added. These fractions were prepared from a schizont-rich culture of P. falciparum (PAM strain at 78% of mature forms) synchronized by several rounds of sorbitol treatment and Plasmagel flotation. The culture was harvested at 9% parasitemia and IRBC were extensively washed with RPMI to remove human serum and secreted soluble proteins. Cells were recounted after washings then serially diluted in RPMI at concentrations equivalent to 5,00,000 to 250 IRBC mL⁻¹. After centrifugation of the diluted samples, the cell pellets were resuspended in an equal volume of buffered water or PBS containing 0.05% SDS. Complete lysis was finally achieved by resuspending the IRBC vigorously, and the samples were centrifuged at 10,000 g for 10 min. at 4°C to eliminate cellular debris. An equivalent preparation of uninfected human erythrocytes was prepared and used as a negative control. For the assay, duplicate samples of 100 μl of the hemolysates were added to the wells and incubated for 2 h. After additionnal washings, bound antigens were detected by incubation with 100 μl/well of a 1:5,000 dilution of biotinylated F1546 antibodies (1 mg.ml⁻²). Detection was achieved by addition of avidin-peroxidase (A3141, Sigma) and TMB microwell Peroxidase substrate (KPL) according to manufacturer recommendations. The reaction was stopped by addition of HCl 2N and O.D. values were read spectrophotometrically at 450 nm. Otherwise indicated all incubations steps were performed at room temperature and three washings were systematically performed with PBS-T.

Electrophoresis and immunoblots. The samples were resuspended in Laemmli sample buffer (Biorad) with or without dithiothreitol, and boiled for 5 min. Samples were electrophoretically separated using the Criterion Precast Gel System on XT Bis-Tris 10% or 4–12% resolving gels (Biorad). The extracts were stained with Commassie brilliant blue or transferred onto nitrocellulose membranes by electrophoresis O/N at 30V. Blots were processed according to standard procedures and blocked with 50 mM Tris, pH 8, containing 0.15 M NaCl, 0.05% Tween 20, 5% non-fat milk. They were further incubated with MAb s or recombinant antibody fragments. Bound antibodies were detected by using an alkaline phosphatase conjugate of anti-mouse IgG whole molecule (Goat Anti-Mouse IgG, S3721, Promega, France) or an anti-mouse IgG (Fab specific)-Alkaline Phosphatase antibody produced in goat (A1293, Sigma, France).
Nucleic acids extraction and cDNA synthesis. Total nucleic acids were extracted from *P. falciparum* infected erythrocytes using the High Pure PCR template preparation kit (Roche Applied Science) following manufacturer's protocol. For each sample, 200 μL of cell suspension were processed yielding a 200 μL final volume of nucleic acids in elution buffer. The sample was then treated by Dnase I Rnase free (Roche Applied Science) to eliminate genomic DNA. After an incubation step of 10 min at 37°C the Dnase was inactivated by boiling for 15 min. Hybridoma cells were grown in DMEM medium supplemented with 20% fetal calf serum. Cells were recovered by centrifugation and the pellet containing approximately 10^7 hybridoma cells was resuspended in 1 ml TRIzol reagent (Invitrogen) and conserved at -80°C. Total RNA was extracted according to manufacturer instructions. Briefly, after thawing the cells, 200 μL of chloroform were added to the sample. After vigorous shaking the tubes were centrifuged at 10,000 g for 15 min at 4°C, RNA was precipitated with isopropyl alcohol and recovered by centrifugation at 10,000 g for 10 min. The supernatant was washed with 75% ethanol and resuspended in 100 μL of RNase free water. The purity of the RNA was adjusted at 1/1,00,00 dilution. Binding was monitored using a BCIP/NBT mixture substrate (S3771, Promega) according to manufacturer recommendations.

Figure 8. Binding specificities of the recombinant FabF1546-H6 as determined by ELISA on parasite and recombinant PfHRP2. In part A, microplate wells were coated with a crude *P. falciparum* soluble extract (protein concentration adjusted to 20 μg.mL^-1^ with PBS) and reacted with two-fold dilutions of a periplasmic extract of HB2151(pF1546) from 1/10 to 1/10,240. In part B, wells were coated with various concentrations of affinity-purified Mal-E-PfHRP2 protein, from 1.5 ng.mL^-1^ to 20 μg.mL^-1^, and reacted with a periplasmic extract of HB2151 (p1546) at a 1/10 dilution. Bound antibodies or fragments were detected with an alkaline phosphatase-conjugated Fab-specific anti mouse IgG. Doted lines correspond to the technical background level (mean blank values + 2SD).
insert from three independent clones was sequenced. All had the same sequence encoding a protein identical to the published 3D7 sequence (embl accession number AL844506.2). The 3D7 P/HRP2 insert was then subcloned into a pMAL-c2X vector (New England BioLabs) between the EcoRI and BamHI sites and used to transform XL1-Blue competent cells. Positive clones were identified by PCR and the construct was verified by restriction analysis and DNA sequencing. The resulting plasmid named pER1, encoded a fusion protein between the MalE protein from E. coli and P/HRP2, MalE-P/HRP2. pER1 was introduced by transformation into the E. coli strain HB2151.29 The production of MalE-P/HRP2 was induced by 0.3 mM IPTG for 2 h according to standard protocol (pMAL™ protein fusion and purification system instruction manual, New England Biolabs).

Amplification and cloning of the cDNA fragments encoding VL-CL and VH-CH1 of hybridomas F1546 and F1110. VL-CL and VH-CH1 sequences were PCR amplified from reversed transcribed hybridoma cDNA by using a panel of degenerate primers containing appropriate restriction sites enabling the various PCR products to be inserted into the expression vector pPE1.30 The sequences encoding the F1546 and F1110 light chains (VL-CL) were amplified in good yields using the CK and LC7 primers containing appropriate restriction sites enabling the various PCR products to be inserted into the expression vector pPE1. The products to be inserted into the expression vector pPE1. Each step of the assembling was verified by restriction analysis and the final constructs were sequenced. In each case, the sequence of the inserted DNA fragments matched the consensus sequences and ORF was as predicted. These constructs were used to transform XL1-Blue competent cells and positive clones were kept at -80°C in glycerol for long term storage. The FabF1546-H6 and FabF1110-H6 were produced in strain HB2151 as described.9,29,30,39 Briefly, the bacteria were grown overnight at 30°C in glucose rich SBD1 medium and further incubated at 22°C until the culture reached OD600 nm = 0.5. The bacteria were then induced with 0.2 mM IPTG for two hours, harvested by centrifugation and treated for 30 min with 1 mg.mL-1 polymyxin B to collect the periplasmic fluid. FabF1546-H6 and FabF1110-H6 were purified from the periplasmic fluids by affinity chromatography on Ni-NTA Spin Columns (Qiagen) according to the manufacturer recommendations.

Direct ELISA assay of the binding properties of FabF1546-H6. Maxisorp™ high-protein binding capacity ELISA plates (Nunc, France) were coated overnight at +4°C with 100 μL of either a crude parasite antigenic extract at a concentration of 20 mg.mL-1 in PBS, or with decreasing quantities of affinity purified MalE-P/HRP2 recombinant protein in PBS. The parasite extract was prepared from the PAM strain. Briefly, the culture was harvested at the schizont stage and washed once in culture medium. Infected red blood cells were re-suspended in Plasmagel to enrich the culture in mature parasite stages. After 30 min at 37°C, supernatants were washed twice in RPMI and the cell pellet was resuspended in buffered water containing a cocktail of protease inhibitors. After two cycles of freezing and thawing, the lysate was finally centrifuged for 10 min at 10,000 g. The supernatant containing water soluble parasite antigens was quantified for protein content by using the Biorad protein assay. The Fab fragments were prepared as periplasmic fluids that resulted from the treatment of the E. coli producing cells with polymyxin B and dialysis against PBS. Plates were saturated for 2 h at RT with 200 μL PBS, pH 7.2, containing 5% bovine serum albumin (BSA). The plates were washed twice with PBS-T. Then, 100 μL of periplasmic fluid, diluted in PBS-T containing 1% BSA (PBS-T-BSA), were dispensed into the wells. The plates were incubated for 1 h at room temperature and washed three times with PBS-T. Binding
of Fabs to antigenic preparations was detected by addition of 100 µL of an Anti-Mouse IgG (Fab specific)-Alkaline Phosphatase antibody produced in goat (A1293, Sigma) at a 1:10,000 dilution in PBS-T. The plates were washed three times and 100 µL of 5’-bromo-4-chloro-3-indolyl-phosphate substrate (BluePhos R substrate system, KPL) was added. The plates were incubated for 10 min after which the enzymatic reaction was stopped. The absorbance of solutions was determined at 650 nm.

Acknowledgements

This work was funded by Sanofi-Aventis and the French Ministry of Research and New Technologies “Fonds Dédié pour Combattre les Maladies Parasitaires” and by the Institut Pasteur International Network. E. Ravaoarisoa was supported by a special grant from the Institut Pasteur International Network. We would like to acknowledge Drs. P.H. David (Institut Pasteur), A. Talarmin and D. Menard (Institut Pasteur de Madagascar) for their continuous support and helpful discussions. We are grateful to Dr. M. Randrianarivelosia for valuable comments on the manuscript. We also thank M. Guilloitte and I. Vigan for their expertise in large scale in vitro production of F. falciparum parasites.

References

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 2005; 434: 214-7.
2. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bull World Health Organ 1988; 66:621-6.
3. Mwangi TW, Ross A, Snow RW, Marsh K. Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. J Infect Dis 2005; 191:1232-9.
4. Olivier M, Develoux M, Chegou Abari A, Louzan L. Presumptive diagnosis of malaria results in a significant risk of mistreatment of children in urban Sahel. Trans R Soc Trop Med Hyg 1991; 85:279-30.
5. Ameso M, Tolhurst R, Barnish G, Bates J. Malaria mini-diagnosis: effects on the poor-and vulnerable. Lancet 2004; 364:1896-8.
6. Zikusooka CM, McIntyre D, Barnes KI. Should countries implementing an artemisinin-based combination malaria treatment policy also introduce rapid diagnostic tests? Malar J 2008; 7:176.
7. WHO. 2000. New perspectives, malaria diagnosis: report of a joint WHO/USAID informal consulta-
tion. Oct 25–27, 1999; WHO/CDS/RRM/2000.14, WHO/MAL/2000.1091, WHO, Geneva (2000).
8. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev 2002; 15:66-78.
9. Wongrichanalai C. Rapid diagnostic techniques for malaria control. Trends Parasitol 2001; 17:307-9.
10. Beadle C, Long GW, Weiss WR, McElroy PD, Maret SM, Oloso AJ, et al. Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet 1994; 343: 564-8.
11. Taylor DW, Voller A. The development and validation of a simple antigen detection ELISA for Plasmodium falciparum malaria. Trans R Soc Trop Med Hyg 1993; 87:29-31.
12. Shiff CJ, Minjas J, Premji Z, The ParaSight-F test: a simple rapid manual dipstick test to detect Plasmodium falciparum infection. Parasitol Today 1994; 10:494-5.
13. Murray CK, Bell D, Gasser RA, Wongrichanalai C. Rapid diagnostic testing for malaria. Trop Med Int Health 2003; 8:876-83.
14. Forney JR, Magill AJ, Wongrichanalai C, Srichaisinthorn J, Bautista CT, Heppner DG, et al. Malaria rapid diagnostic devices: performance characteristics of the ParaSight F device determined in a multi-site field study. J Clin Microbiol 2001; 39:2884-90.
15. Payne D, Bhat E, Evans CB, Taylor DW. Identification of Plasmodium falciparum histidine-rich protein 2 in the plasma of humans with malaria. J Clin Microbiol 1991; 29:1629-34.
16. Desakorn V, Dondorp AM, Silamut K, Pongvoravipnoy W, Sahassananda D, Choratichai K, et al. Stage-dependent production and release of histidine-rich protein 2 by Plasmodium falciparum. Trans R Soc Trop Med Hyg 2005; 99:517-24.
17. Howard RJ, Uni S, Aikawa M, Aley SB, Leech JH, Lew AM, et al. Secretion of a malarial histidine-rich protein (PI HRP II) from Plasmodium falciparum-infected erythrocytes. J Cell Biol 1986; 103:1269-77.
18. WHO. 2008. Malaria Rapid diagnostic test perform-
ance. Results of WHO product testing of malaria RDTs: Round 1. www.wpro.who.int/NN/edondyles/ EDN1B0ED0-B812-4B08-8400-31A926356C40/ OMSFIND RapportMalaria20090511-625.pdf.
19. WHO. 2006. Towards quality testing of malaria rapid diagnostic tests: evidence and methods. WHO, western Pacific Region. Manila, Philippines.
20. Bell DF, Wongrichanalai C, Barnwell JW. Ensuring quality and acceptor for malarial diagnostic: how can it be achieved? Nat Rev Microbiol 2006; 6:7-20.
21. WHO. Sources and Prices of Selected Products for the Prevention, Diagnosis and treatment of malaria; A joint WHO RBM, UNICEF, UNAIDS, PSI, MSH project. WHO. 2008. Malaria Rapid diagnostic test perfor-
mance. Results of WHO product testing of malaria RDTs: Round 1. www.wpro.who.int/NN/edondyles/ EDN1B0ED0-B812-4B08-8400-31A926356C40/ OMSFIND RapportMalaria20090511-625.pdf.
22. WHO. 2002. Diagnosis and management of severe malaria. Jun 2002; Trial edition. www.malaria.org.za/ Case/cm26.pdf.
23. Baker J, McCarthy J, Garton M, Kyle DE, Belizario V, Luchavez J, et al. Generic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance of PFHRP2-based rapid diagnostic tests. J Infect Dis 2005; 192:870-7.
24. Zerpa NC, Wide A, Noda J, Bermudez H, Pabon R, Noya OO. Immunogenicity of synthetic peptides derived from Plasmodium falciparum proteins. Exp Parasitol 2006; 113:227-34.
25. Versteege I, Ment PF. Development of a stable positive control to be used for quality assurance of rapid diagnostic tests for malaria. Diagn Microbiol Infect Dis 2009; 64:256-60.
26. Smith KA, Nelson PN, Warren P, Ashley SJ, Muggen GW, Greenman J. Demystified recombinant antibodies. J Clin Pathol 2004; 57:912-7.
27. Monostier E, Bedouelle H. Improving the stability of an antibody variable fragment by a combination of knowledge-based approaches: validation and mecha-
nisms. J Mol Biol 2006; 362:580-93.
28. Fondeur B, Bonnefoy S, Mercereau-Puijalon O. In vivo and in vitro derived Yale Alto lines of Plasmodium falciparum are genetically unrelated. Mol Biochem Parasitol 1991; 47:167-78.
29. Trager W, Jensen JB. Human malaria parasites in con-
tinuous culture. Science 1976; 193:673-5.
30. Douyé JC, Guandoou JL, Tokou H, Maretelli M, Bonnefoy S, Mercereau-Puijalon O. Characterisation of the binding sites of monoclonal antibodies react-
ing with the Plasmodium falciparum rhoptry protein RhopH3. Mol Biochem Parasitol 1997; 85:149-59.