Functional Comparison of Plasmodium falciparum Transmission-Blocking Vaccine Candidates by the Standard Membrane-Feeding Assay

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Recently, there has been a renewed interest in the development of transmission-blocking vaccines (TBV) against Plasmodium falciparum malaria. While several candidate TBVs have been reported, studies directly comparing them in functional assays are limited. To this end, recombinant proteins of TBV candidates Pfs25, Pfs230, and PfHAP2 were expressed in the wheat germ cell-free expression system. Outbred CD-1 mice were immunized twice with the antigens. Two weeks after the second immunization, IgG levels were measured by enzyme-linked immunosorbent assay (ELISA), and IgG functionality was assessed by the standard membrane-feeding assay (SMFA) using cultured P. falciparum NF54 gametocytes and Anopheles stephensi mosquitoes. All three recombinant proteins elicited similar levels of antigen-specific IgG judged by ELISA. When IgGs purified from pools of immune serum were tested at 0.75 mg/ml in the SMFA, all three IgGs showed 97 to 100% inhibition in oocyst intensity compared to control IgG. In two additional independent SMFA evaluations, anti-Pfs25, anti-Pfs230, and anti-PfHAP2 IgGs inhibited oocyst intensity in a dose-dependent manner. When all three data sets were analyzed, anti-Pfs25 antibody showed significantly higher inhibition than the other two antibodies (P < 0.001 for both), while there was no significant difference between the other two (P = 0.15). A proportion of plasma samples collected from adults living in an area of malaria endemicity in Mali recognized Pfs230 and PfHAP2. This is the first study showing that the HAP2 protein of P. falciparum can induce transmission-blocking antibody. The current study supports the possibility of using this system for a comparative study with multiple TBV candidates.

Global malaria deaths, mostly caused by Plasmodium falciparum, dropped from an estimated 1.8 million in 2005 to 1.2 million in 2010 (1). This reduction in mortality has been largely attributed to the expanded application of antimalarial drugs, insecticide-treated nets, and indoor insecticide spraying. Due to increased resistance of parasites to antimalarials and of mosquito vectors to insecticides, vaccines and other new control measures will be required to achieve malaria control and elimination. In recent years, there has thus been a renewed interest in the development of transmission-blocking vaccines (TBVs). TBVs are designed to induce antibodies in human hosts against sexual-stage malaria antigens or to antigens found in the mosquito vector. TBV-induced antibodies, which are ingested by Anopheles mosquitoes along with gametocytes in the blood, act by inhibiting parasite development in the mosquito.

The Malaria Eradication Research Agenda (malERA) consultative group recently proposed the concept of a “vaccine that interrupts malaria transmission” (VIMT) (2). In addition to the “classical” TBVs, VIMTs include preerythrocytic and asexual blood stage vaccines that may indirectly reduce parasite transmission. Among the preerythrocytic vaccines, RTS,S/AS01 is the most advanced vaccine, and it has recently been evaluated in a large phase 3 trial in African children. However, the vaccine efficacy in reducing the incidence of clinical malaria in 6- to 12-week-old children over 14 months was only 30% (3), suggesting that additional approaches will be necessary to control malaria.

Of the classical TBV candidates, only P. falciparum surface protein 25 (Pfs25) and the Plasmodium vivax homolog Pvs25 have been tested in phase 1 clinical trials (4, 5). These existing TBV candidates and formulations were not optimal because they induced insufficient levels of functional antibodies in humans and/or showed some safety concerns (the specific antigen-adjuvant combination, not the antigen per se, was thought to cause the adverse reactions) (5). Therefore, further development of effective and safe TBVs is necessary.

The standard membrane-feeding assay (SMFA) has been widely used to assess the transmission-blocking potential of test antibodies. In this assay, a mixture of cultured P. falciparum gametocytes and test antibodies is fed to Anopheles mosquitoes through a membrane-feeding apparatus, and approximately 1 week later the mosquitoes are dissected to enumerate oocysts in their midguts. Multiple antigens have been identified as TBV candidates (reviewed in reference 6); however, few have directly compared them in functional assays, such as the SMFA. We previously showed that recombinant Pfs25 and Pfs230 proteins, produced in the wheat germ cell-free (WGCF) expression system...
system, could elicit functional antibodies as assessed in the SMFA (7, 8). In the present report, the PHAP2 recombinant protein was also expressed in the WGCFS system, and these three candidates were compared head-to-head by a qualified SMFA (9). The protein of a rodent ortholog, Plasmodium berghei HAP2, has previously shown to induce functional antibody (10), but this is the first study showing the transmission-blocking activity of anti-PHAP2 antibody in P. falciparum. While all three IgGs showed significant inhibition in the SMFA, the anti-Pfs25 IgG exerted the greatest effect among them.

**MATERIALS AND METHODS**

**Construct design and production and purification of recombinant proteins.** Amino acid sequences for Pfs25 (amino acids [aa] 24 to 193; PF3D7_0209000), Pfs230 (aa 443 to 1132, Pfs230C; PF3D7_0209000), and PHAP2 (aa 195 to 684; PF3D7_1014200) were obtained from the NCBI protein database (Table 1). For Pfs25, full-length sequences without the N terminus signal peptide and C-terminal glycosylphosphatidylinositol (GPI) anchor signal sequences were used. For Pfs230, region C (Pfs230C), against which antibodies have been shown to inhibit parasite development, was used (8, 11). For PHAP2, a region covering the P. berghei ortholog (aa 355 to 609) (10) was used, as the antibody against the fragment of P. berghei HAP2 has been shown to inhibit parasite development (10). The antigen sequences were codon optimized for expression in wheat (GenScript, Piscataway, NJ), and the Xhol restriction site with the start codon at the N terminus and the hexa-histidine tag at the C terminus.

**IgG purification.** IgGs from mouse serum (pooled by antigen group) were purified using protein G affinity chromatography (Pierce, Rockford, IL) according to the manufacturer’s instructions and adjusted to a final concentration of 8 mg/ml in phosphate-buffered saline (PBS). Based on the ELISA result, serum samples from individual mice showing titers less than the mean minus 3 SD of the group of mice were excluded from the pool of serum used for IgG purification (one mouse each in the Pfs230C and PHAP2 groups).

**SMFA.** The standardized methodology for performing the enzyme-linked immunosorbent assay (ELISA) has been described previously (12). Plasma samples from Malian adults (n = 45) were collected from a study approved by the Ethics Committee of the Faculty of Medicine, Pharmacy and Odontostomatometry, University of Bamako, and the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (NIAID) (13). Individual written informed consent was obtained from all participants. The study is registered with Clinicaltrials.gov, number NCT00669084. American sera (n = 24) were purchased from Interstate Blood Bank, Inc. (Memphis, TN). The Malian and American adult samples were tested at 1:100 dilution, and the direct OD value was used as a final readout, instead of ELISA units. An individual Malian sample with an OD value greater than the mean plus 3 standard deviations (SD) of the pool of American samples was considered a “positive” sample.

**Animal immunization.** The animal study was performed in compliance with National Institutes of Health (NIH) guidelines and under an Animal Care and Use Committee-approved protocol (LMVR 10E). CD-1 mice (n = 10 per group) were immunized intraperitoneally with 25 μg recombinant protein formulated with Montanide ISA720 (SEPPIC Inc., Fairfield, NJ) on day zero. The mice were then booster-immunized subcutaneously with 10 μg recombinant protein formulated with Montanide ISA720 on day 28. The serum samples were collected on days 0 and 42. Due to a technical problem, two serum samples were not collected on day 42 for one mouse each in the Pfs25 and HisGST groups.

**ELISA.** The standardized methodology for performing the enzyme-linked immunosorbent assay (ELISA) has been described previously (12). The absorbance of each test sample was converted into ELISA units using a standard curve generated by serially diluting the standard in the same plate. The ELISA unit value of a standard was assigned as the reciprocal of the dilution giving an optical density at 405 nm (OD_{405}) of 1 in a standardized assay. The minimal detection level of IgG in this study was 26 ELISA units.

Plasma samples from Malian adults (n = 45) were collected from a study approved by the Ethics Committee of the Faculty of Medicine, Pharmacy and Odontostomatometry, University of Bamako, and the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Diseases (NIAID) (13). Individual written informed consent was obtained from all participants. The study is registered with Clinicaltrials.gov, number NCT00669084. American sera (n = 24) were purchased from Interstate Blood Bank, Inc. (Memphis, TN). The Malian and American adult samples were tested at 1:100 dilution, and the direct OD value was used as a final readout, instead of ELISA units. An individual Malian sample with an OD value greater than the mean plus 3 standard deviations (SD) of the pool of American samples was considered a “positive” sample.

**Statistical analysis.** ELISA units among the three groups were compared by a Kruskal-Wallis test. Percent inhibition of mean oocyst intensity (PIm) was calculated by the following formula: 100 × [1 – (mean number of oocysts in the test group)/(mean number of oocysts in the HisGST group)], where the confidence interval of the percent inhibition is estimated from the model described below. Similarly, percent inhibition of prevalence (PIp) was calculated as follows: 100 × [1 – (proportion of mosquitoes with any oocysts in the test group)/(proportion of mosquitoes with any oocysts in the HisGST group)].

We used a negative binomial model with zero inflation (14). When there were no observed oocysts in any of the dissected mosquitoes (i.e., the anti-Pfs25 IgG tested at 0.75 mg/ml in the first and second feeding experiments), we changed the oocyst count to 1 for the first of the mosquitoes in that group so the model would converge and give conservative P values and lower confidence limits (but we kept the PIm estimate as 100%). We tested for significance of PIm using that model and for PIp by repeatedly

<table>
<thead>
<tr>
<th>Group</th>
<th>Construct</th>
<th>Length (aa)</th>
<th>Purification tag</th>
<th>Purification</th>
</tr>
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<tbody>
<tr>
<td>HisGST</td>
<td></td>
<td>241 GST</td>
<td>Affinity + size exclusion</td>
<td></td>
</tr>
<tr>
<td>Pfs25</td>
<td>24–193</td>
<td>170 His</td>
<td>Affinity + size exclusion</td>
<td></td>
</tr>
<tr>
<td>Pfs230C</td>
<td>443–1132</td>
<td>690 GST</td>
<td>Affinity + size exclusion</td>
<td></td>
</tr>
<tr>
<td>PHAP2</td>
<td>195–684</td>
<td>490 GST</td>
<td>Affinity</td>
<td></td>
</tr>
</tbody>
</table>

a Amino acid residue numbers.

b GST, GST tag at the N terminus; His, hexa-histidine tag at the C terminus.

c Affinity, affinity chromatography; size exclusion, gel filtration chromatography.
Immunogenicity of TBV candidates. CD-1 mice (n = 10 per group) were immunized with Pf625, Pf6230C, or PHAP2, and their levels of specific IgG for homologous antigen were determined by ELISA. IgG responses below the limit of detection (26 ELISA units) were assigned a value of 26 ELISA units. Box and whisker plots (10th to 90th percentiles) are shown.

FIG 3 Three TBV candidates are equally immunogenic. CD-1 mice (n = 10 per group) were immunized with Pf625, Pf6230C, or PHAP2, and their levels of specific IgG for homologous antigen were determined by ELISA. IgG responses below the limit of detection (26 ELISA units) were assigned a value of 26 ELISA units. Box and whisker plots (10th to 90th percentiles) are shown.
second SMFA and at 0.75, 0.38, 0.19, and 0.09 mg/ml in the third SMFA. When the square root of total IgG level was plotted against the ratio of the mean oocyst count in control and test samples (log scale), the data showed an approximate linear relationship (Fig. 4), as seen previously for an anti-Pfs25 monoclonal antibody (9).

While all three anti-TBV IgGs showed significant dose-dependent inhibition in SMFA (Holm’s adjusted \( P < 0.001 \) for all), anti-Pfs25 IgGs showed significantly higher inhibition than anti-Pfs230C and anti-PfHAP2 IgGs (Holm’s adjusted \( P < 0.001 \) for both). Anti-Pfs230C and anti-PfHAP2 IgGs showed no significant difference in transmission-blocking activity (Holm’s adjusted or unadjusted \( P = 0.17 \)). These data indicate that Pfs25 elicits the most effective IgG response of the three TBV candidates expressed in the WGCF system.

### IgG responses to three TBV candidates in malaria-exposed Malian adults.

Finally, we tested whether naturally acquired antibodies in malaria-exposed Malian adults recognized the three TBV candidates that successfully induced transmission-blocking IgGs in mice. As expected, Malian adult IgGs, except 1 (2%) of 45 tested, do not recognize Pfs25 (Fig. 5A), which is not expressed by gametocytes in humans (16). On the other hand, 23 (51%) and 13 (29%) samples recognized Pfs230C (Fig. 5B) and PfHAP2.

### Table 2: Transmission-blocking activities of pooled IgGs from mice immunized with TBV candidates

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean no. (SD) of oocysts(^a)</th>
<th>PLm (95% CI)(^b)</th>
<th>( P ) value of oocyst(^c)</th>
<th>No. of mosquitoes without oocysts/total no. of mosquitoes examined</th>
<th>PLp(^d)</th>
<th>( P ) value of incidence(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HisGST</td>
<td>14.5 (9.1)</td>
<td>NA</td>
<td>NA</td>
<td>1/20</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pfs25</td>
<td>0.0 (0.0)</td>
<td>100 (98.100)</td>
<td>&lt;0.001</td>
<td>20/20</td>
<td>100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pfs230C</td>
<td>0.4 (0.0)</td>
<td>98 (95.99)</td>
<td>&lt;0.001</td>
<td>17/20</td>
<td>84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PfHAP2</td>
<td>0.5 (0.6)</td>
<td>97 (94.99)</td>
<td>&lt;0.001</td>
<td>12/20</td>
<td>58</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\( ^a \)All purified IgGs were tested at 0.75 mg/ml with complement. NA, not applicable.

\( ^b \)Per mosquito midgut.

\( ^c \)Percent inhibition of mean oocyst intensity (PLm) and 95% CI compared to the HisGST group. The 95% CIs were calculated from the zero-inflation negative binomial model (9).

\( ^d \)\( P \) values were calculated from the zero-inflation negative binomial model (9), and Holm’s corrected \( P \) values are shown.

\( ^e \)Percent inhibition of prevalence (PLp) compared to the HisGST group.

\( ^f \)The ratio of the number of mosquitoes with oocysts to the number without oocysts of each test group was compared with that of the HisGST group by Fisher’s test, and Holm’s corrected \( P \) values are shown.

### Figure 4: Dose-dependent inhibition of oocyst formation by anti-TBV IgGs in the SMFA.

Purified anti-Pfs25, anti-Pfs230C, and anti-PfHAP2 IgGs were tested at 0.75 mg/ml in the first SMFA, at 0.75, 0.15, and 0.03 mg/ml in the second SMFA, and at 0.75, 0.38, 0.19, and 0.09 mg/ml in the third SMFA. Mean oocyst numbers of anti-HisGST IgG tested at 0.75 mg/ml (14.5, 15.2, and 48.6 oocysts per mosquito in the first, second, and third SMFAs, respectively) were used as controls. The square root of total IgG level is shown on the x-axis. The ratios of mean oocyst counts in control and test samples are plotted on a log scale along the left y-axis, and the associated PLm value is plotted along the right y-axis. Data points from the same SMFA are connected by dotted lines for the second and third SMFAs.

### Figure 5: IgG responses to three TBV candidates in malaria-exposed Malian adults.

Malian adult plasma samples (\( n = 45 \)) and U.S. adult sera (\( n = 24 \)) were tested at 1:100 dilution in ELISA. Reverse cumulative distribution plots of Malian samples are shown for Pfs25 (A), Pfs230C (B), and PfHAP2 (C). The dotted line in each panel represents the OD value of the mean plus 3 SD of U.S. adult plasma samples for each protein.
DISCUSSION

While several studies have identified TBV candidates, few have directly compared them in functional assays. To this end, recombinant proteins of TBV candidates Pfs25, Pfs230C, and PfHAP2 were expressed in the WGCF system and tested head-to-head in SMFAs for their ability to induce transmission-blocking antibodies in mice. This is the first study showing that the HAP2 protein of *P. falciparum* (like the HAP2 homolog of *P. berghei*) can induce transmission-blocking antibodies. When the anti-Pfs25, anti-Pfs230C, and anti-PfHAP2 IgGs were compared, the anti-Pfs25 IgG showed the highest inhibition in SMFA.

In our recent attempt to express cDNA clones from *P. falciparum* sporozoites, merozoites, and gametocytes in a high-throughput WGCF system, we found that 84% of them (478/567) yielded soluble protein products (17). Additional work revealed that the WGCF expression system achieves a higher yield of full-length proteins than does an *Escherichia coli*-based cell-free system (18). Moreover, our success in identifying novel malaria vaccine candidates (7, 8, 19, 20) suggests that recombinant proteins produced in the WGCF system are able to adopt the proper conformations and display the same antigenic epitopes (maybe not all, but at least some of the epitopes) as their native counterparts. Therefore, we believe that the proteins expressed by the WGCF system are preferable for proper assessment and identification of a very large number of immunodominant malaria vaccine candidate antigens.

Since the WGCF system can generate a great variety of vaccine candidate antigens and produce large quantities of soluble proteins in a very short time, we decided to use this system in the present study.

SMFA is one of a few biological assays that are widely used to assess the transmission-blocking potential of test antibodies in both preclinical and clinical vaccine development. As currently performed, however, the assay has relatively large intra- and interassay variability, especially when antibodies with relatively weak transmission-blocking activity (i.e., less than 80% inhibition) are tested (9). Since it is impossible to achieve more than 100% inhibition, by definition, the dynamic range of this assay is only 80 to 100% inhibition. Consequently, multiple SMFAs using multiple antibody concentrations may be required to evaluate whether one test antibody is better than another, as performed in this study. Since the dynamic range of the SMFA readouts is narrow, we did not test for additive or synergistic effects between transmission-blocking IgGs in this study. Demonstrating significant additive or synergistic effects, given the assay’s variability, is likely to be difficult, unless the effect is very strong or the same IgGs (and a combination of IgGs) are tested in multiple feeds. Further studies to evaluate whether immunization with a mixture of these antigens induces more-effective IgG responses than immunization with a single antigen are anticipated.

One significant limitation of SMFA-based studies is that the transmission-blocking activity measured by SMFA has not been shown to predict TBV efficacy in the field—at either individual or at population levels, since none of the TBV has reached a phase 3 clinical trial to evaluate field efficacy. However, no alternative biological assays, other than direct membrane-feeding assay (where patient blood instead of cultured parasites is the source of gametocytes for the SMFA), are available for preclinical TBV development at this time. Therefore, we believe head-to-head comparisons of transmission-blocking activity using the SMFA are valuable approaches for comparison of multiple TBV candidates. There is a limitation also in the WGCF system. We originally planned to compare five leading TBV candidates, including Pfs48/45 and anopheline alanyl aminopeptidase N (AnAPN1), both of which are potential TBV candidates (21, 22). While both antigens were solubly expressed and equally immunogenic as judged by ELISA, we observed no inhibition in SMFA using antibodies to both candidates under the same study conditions. IgG levels were −42 (P = 0.129) and −6 (P = 0.755) for the Pfs48/45 and AnAPN1 groups, respectively, when the IgGs were tested at 0.75 mg/ml in a single feeding experiment. Since the original statistical analytic plan was to test five groups, in this report we are presenting Holm’s corrected P values for five groups, which are more-conservative estimates. Although there are several possible explanations for the discrepancy from the previous studies, one is that properly folded proteins may not have been efficiently produced in this system. Some modification in the WGCF expression system may be required for certain proteins.

In summary, recombinant Pfs25, Pfs230C, and PfHAP2 proteins expressed in the WGCF system induced functional antibodies in mice. Among these proteins, Pfs25 elicited the highest levels of transmission-blocking antibody. These SMFA results support further development of the TBV candidates.

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REFERENCES


