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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.parint.2009.02.002.

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Single amino acid substitution in *Plasmodium yoelii* erythrocyte ligand determines its localization and controls parasite virulence

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The major virulence determinant of the rodent malaria parasite, *Plasmodium yoelii*, has remained unresolved since the discovery of the lethal line in the 1970s. Because virulence in this parasite correlates with the ability to invade different types of erythrocytes, we evaluated the potential role of the parasite erythrocyte binding ligand, *Py*EBL. We found 1 amino acid substitution in a domain responsible for intracellular trafficking between the lethal and nonlethal parasite lines and, furthermore, that the intracellular localization of *Py*EBL was distinct between these lines. Genetic modification showed that this substitution was responsible not only for *Py*EBL localization but also the erythrocyte-type invasion preference of the parasite and subsequently its virulence in mice. This previously unrecognized mechanism for altering an invasion phenotype indicates that subtle alterations of a malaria parasite ligand can dramatically affect host–pathogen interactions and malaria virulence.

dense granule | invasion | malaria | microneme | transfection

The rodent malaria parasite *Plasmodium yoelii yoelii* has been widely studied to understand the interactions between the malaria parasite and the host cell (1). The nonlethal 17X line mainly infects young erythrocytes (reticulocytes), whereas the lethal 17XL and YM lines infect a wide range of erythrocytes. These lines have previously been studied to identify the genetic determinants of virulence (2, 3). These differences in erythrocyte invasion preference suggest the possible involvement of a parasite ligand that recognizes erythrocyte surface receptors; however, the actual molecular basis of the observed invasion preference differences remains unclear.

Erythrocyte invasion by the malaria merozoite is a multistep process, initiated by reversible binding to the erythrocyte surface, followed by the establishment of a tight junction between the apical end of the merozoite and erythrocyte surface and the subsequent movement of the merozoite into the nascent parasitophorous vacuole. Each step involves specific interactions between parasite ligands and erythrocyte receptors. Among the ligands of malaria parasites, the best characterized is a type I integral transmembrane protein encoded by the *ebl* (*erythrocyte-binding-like*) gene family. Upon release from the micronemes, EBL proteins recognize erythrocyte receptors and initiate the formation of the tight junction. The importance of EBL in malaria virulence is exemplified in the human malaria parasite *Plasmodium vivax*, which uses an EBL orthologue, *Pv*DBP, to recognize the Duffy antigen on the erythrocyte surface. Because the parasite is apparently unable to use an alternative invasion pathway, individuals in whom the Duffy antigen is not expressed on the erythrocyte surface are completely resistant to *P. vivax* (4, 5). Because of this dramatic association between the disruption of a host–pathogen interaction and protection against a malaria parasite, *Pv*DBP and the *Plasmodium falciparum* EBL orthologue, EBA-175, have been targeted for vaccine development (6).

EBL proteins possess 2 Cys-rich regions conserved among EBL orthologues. The N-terminal Cys-rich region named the DBL (Duffy-binding-like) domain or region 2 (7) recognizes a specific erythrocyte surface receptor. The C-terminal Cys-rich region named the C-cys domain or region 6 is located adjacent to the transmembrane domain, and the number and location of Cys residues are well conserved among known *Plasmodium* species. Region 6 exhibits structural similarity to the KIX-binding domain of the coactivator CREB-binding protein (8) and has been proposed to be a protein trafficking signal for transportation to the micronemes (9). Here we report a single nonsynonymous nucleotide substitution in the *pyebl* gene between lethal and nonlethal lines of *P. yoelii* and show the effect of this substitution on the intracellular localization of EBL, erythrocyte-type preference, and consequently virulence of *P. yoelii*.

Results

To investigate differences in EBL between lethal and nonlethal *P. yoelii* lines, we compared sequences from a variety of malaria parasite species and *P. yoelii* lines 17X, 17XL, and YM. We found 1 nonsynonymous nucleotide substitution in region 6 between the nonlethal 17X and lethal 17XL lines in the entire ORF (Fig. 1). The nonlethal 17X line possesses 8 conserved Cys residues that form 4 disulfide bridges (8), whereas the lethal 17XL line possesses an Arg instead of Cys at the second Cys position. This substitution was also found in another lethal line, "YM" (2), which originated independently from the 17X line during serial passage (3). All *Plasmodium* EBL orthologues for which protein expression was validated possess 8 conserved Cys residues in this region, further indicating that these Cys residues play an important role (supporting information Fig. S1). Thus the observed substitution from Cys to Arg is likely to abolish the native conformation of region 6.

EBL Localizes in the Dense Granules in *P. yoelii* Line 17XL. We raised specific polyclonal and monoclonal antibodies against *Py*EBL

Author contributions: H.O., O.K., and M. Torii designed research; H.O., A.T., M. Tachibana, H.I., and S.T. performed research; T.T. contributed new reagents/analytic tools; H.O., O.K., and M. Torii analyzed data; and H.O. and O.K. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The data reported in this article have been deposited in the GenBank/European Molecular Biology Laboratory/DNA Data Base in Japan databases (accession nos. AB430781–AB430789).

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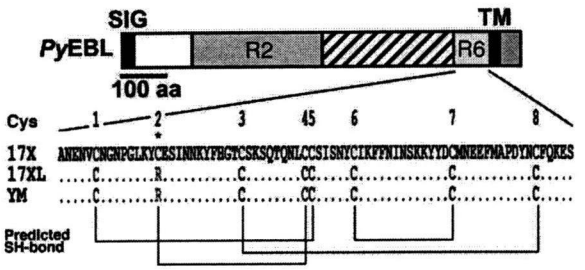

Fig. 1. Schematic structure of *P. yoelii* EBL (*Py*EBL). SIG, TM, R2, and R6 indicate the putative endoplasmic reticulum transporting signal, the transmembrane region, region 2, and region 6, respectively. Amino acid alignment of *Py*EBL from 17X, 17XL, and YM lines are shown below. Eight conserved Cys residues that form disulfide bridges (Predicted SH-bond) and the substitution from Cys to Arg (*) are indicated.

and performed Western blot analysis. The *Py*EBL protein was detected as a 110-kDa band in both the 17X and 17XL lines (Fig. 2*A*). The intracellular localization of *Py*EBL in both the 17X and 17XL lines was compared by indirect immunofluorescent assay

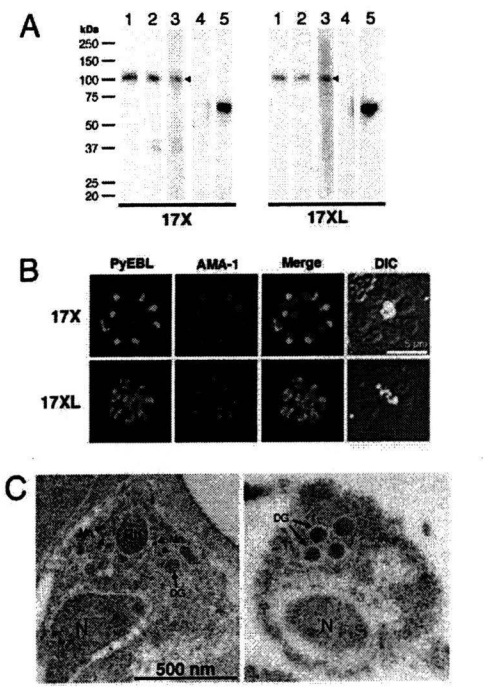

Fig. 2. Western blot analysis and *Py*EBL localization in *P. yoelii* schizont by immunostaining. (*A*) Western blot analysis with mAb 5B10 (lane 1), mAb 1G10 (lanes 2), and mouse serum (lane 3) specific for *Py*EBL against purified *P. yoelii* schizont extracts. A 110-kDa band was detected in both 17X and 17XL lines, with no significant difference in the protein expression level (arrowheads). This band was not detected by normal mouse serum (lane 4). Anti-AMA1 serum detected a 66-kDa band at similar levels (lane 5). (*B*) *P. yoelii* schizonts were incubated with mAb 5B10 (PyEBL), rabbit anti-AMA1 serum (AMA1), and DAPI (blue) for nuclear staining. Schizonts labeled with anti-*Py*EBL (5B10) were stained with FITC secondary antibody (green). Anti-AMA1 were stained with Alexa-546 secondary antibody (red). DIC images are shown in the right-hand column. The 17X line shows apical *Py*EBL signal colocalized with AMA1, but the region 6–substituted 17XL line shows diffused staining that does not colocalize with AMA1. (*C*) Immunoelectron microcopy was carried out for resin-embedded *P. yoelii* 17X and 17XL lines with anti-*Py*EBL mouse serum and secondary antibody conjugated with gold particles. *Py*EBL was detected in the micronemes (arrowheads) of the 17X line, but in the 17XL line it was located in the dense granules (arrows). N, nucleus; Mn, microneme; DG, dense granule; Rh, rhoptry.

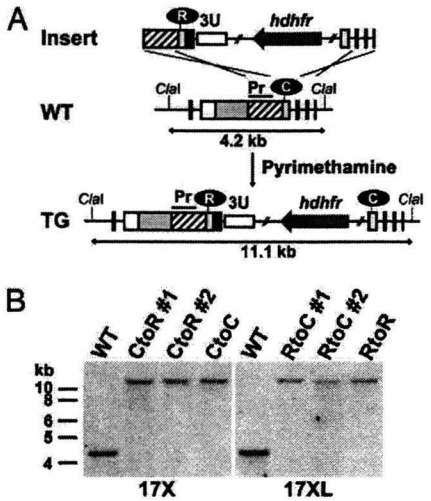

Fig. 3. Amino acid replacement of *Py*EBL region 6 second cysteine location by targeted recombination. (*A*) Schematic representation of the WT and modified (TG) *pyebl* gene loci. The replacement cassette (Insert) was inserted into the *pyebl* gene locus by double-crossover recombination. In this schematic, the second Cys in region 6 was replaced with Arg in the 17X line to generate 17X-CtoR. Other transgenic lines were generated in a similar fashion. ClaI restriction sites and the expected size of the DNA fragment after ClaI digestion are shown. Pr, probe region used in Southern blot analysis. (*B*) Southern blot analysis of the *pyebl* gene locus in WT and transgenic parasite lines derived from *P. yoelii* 17X and 17XL. The absence of the 4.2-kb WT band and the presence of an 11.1-kb band indicate that the *Py*EBL locus was modified in all transgenic clones.

(IFA) using specific antibodies against *Py*EBL (Fig. S2). In the 17X line, *Py*EBL localized to the apical end of each merozoite in both the segmented schizont-stage parasite and individual merozoites, where it colocalized with AMA1, a known microneme protein, under immunofluorescent microscopy (Fig. 2*B*). However, in the 17XL line *Py*EBL did not colocalize with AMA1 at the apical end of merozoites and showed a more diffused but granular distribution in comparison with parasites of the 17X line (Fig. 2*B*). Diffused localization of *Py*EBL was also observed in parasites of the YM line (Fig. S3). Immunoelectron microscopy revealed that *Py*EBL localized in micronemes in the 17X line as reported for *P. falciparum* and *Plasmodium knowlesi* (10, 11). In the 17XL line, however, *Py*EBL localized not in the microneme but in another microorganelle—the dense granules (12) (Fig. 2*C* and Fig. S4).

Because there seems to be only 1 copy of *Py*EBL in the genomes of both lines (Fig. S5), and significant differences were not observed in the level of transcription and protein expression between the 17X and 17XL lines (Fig. 2*A* and Fig. S6), the location of EBL seems to be the most significant difference between them.

Genetic Replacement of Arg and Cys in Region 6 Alters EBL Localization. To evaluate whether the Arg substitution at the second Cys position is responsible for the altered trafficking of *Py*EBL, we exchanged Cys and Arg in the 17X and 17XL lines by genetic modification (17X-CtoR and 17XL-RtoC). The parasites were also transfected with control constructs that do not alter the region 6 amino acid sequence (17X-CtoC and 17XL-RtoR) (Fig. 3*A*). Each of the transgenic parasites was evaluated for the correct integration of the constructs to the *pyebl* gene locus by specific PCR analysis followed by sequencing of the PCR-amplified products (not shown) and Southern blot analysis (Fig. 3*B*).

In the 17X line, replacement of Cys with Arg (17X-CtoR) altered the *Py*EBL localization from an apical pattern to a

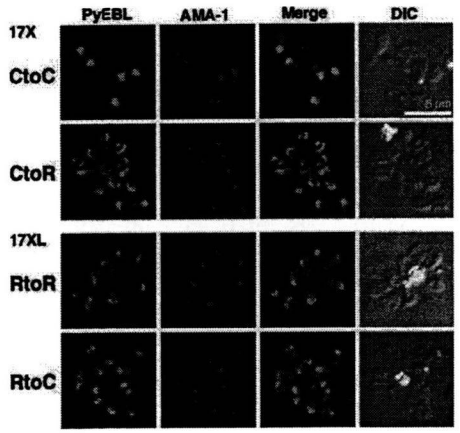

Fig. 4. Replacement of Cys to Arg in region 6 altered subcellular localization of *Py*EBL. Schizonts of transgenic parasite lines were incubated with mAb 5B10 (PyEBL), rabbit anti-AMA1 serum (AMA1), and DAPI (blue) for nuclear staining. DIC images are shown in the right-hand column. In the 17X background, control (CtoC) shows an apical *Py*EBL signal colocalized with AMA1, but replaced (CtoR) shows a 17XL pattern. Inversely, 17XL background control (RtoR) shows a diffused nonapical pattern, but replaced to cysteine (RtoC) shows an apical signal colocalized with AMA1.

nonapical diffused pattern, and *Py*EBL did not colocalize with AMA1. Furthermore, the replacement of Arg with Cys in the 17XL line (17XL-RtoC) altered the *Py*EBL localization from a nonapical diffused pattern to an apical pattern. Control parasites did not display altered *Py*EBL localization (Fig. 4). These results confirm that the observed substitution from Cys to Arg is responsible for the altered localization of *Py*EBL from micronemes to dense granules in the 17XL line.

EBL Localization Alters Erythrocyte-Type Preference and Course of Infection. To determine whether altered localization of *Py*EBL affects erythrocyte-type invasion preference, infected erythrocytes were examined by microscopy, and a selectivity index (SI) was obtained by calculating multiple parasite infection of single erythrocytes for each parasite line on postinfection day 3 in mice (13). We found that 17XL-RtoC predominantly invaded reticulocytes in the same way as the nonlethal 17X line. The SI of the 17XL line (2.38) was increased in 17XL-RtoC ($\approx$35; $P < 0.001$). On the other hand, 17X-CtoR was able to invade a variety of ages of erythrocytes, including mature erythrocytes, comparable to the lethal 17XL line, with the SI of the 17X line (16.78) reduced in 17X-CtoR ($\approx$4; $P < 0.001$; Table 1). These results demonstrate that the localization of *Py*EBL is responsible for the erythrocyte-type preference of the parasite.

Table 1. Selectivity index of WT and transgenic *Plasmodium yoelii* lines

Parasite	*n*	Selectivity index (range)
17X-CtoR 1	5	3.87 (1.86–5.32)
17X-CtoR 2	5	4.25 (2.38–7.97)
17X-CtoC	5	23.53 (16.49–36.00)
17X	5	16.78 (7.60–24.99)
17XL-RtoC 1	5	34.35 (29.18–38.05)
17XL-RtoC 2	5	35.99 (29.97–42.72)
17XL-RtoR	5	1.31 (0.57–2.13)
17XL	5	2.38 (1.58–3.75)

Selectivity indices were calculated from parasitized Giemsa-stained thin blood films collected from each infection.

Because erythrocyte-type preference frequently correlates with virulence in malaria parasites, we further analyzed the transgenic *P. yoelii* parasites for differences in the course of infection and survival of parasite-infected mice. Mice infected with the 17XL-RtoC line developed significantly lower parasitemias compared with the parental 17XL and control 17XL-RtoR lines (Fig. 5*A*), with 100% survival (Fig. 5*C*), whereas all mice infected with 17XL and 17XL-RtoR lines died by day 7 (Fig. 5*C*). The pattern observed for the 17XL-RtoC line was identical to that observed for the nonlethal 17X line. Thus, trafficking of *Py*EBL to the micronemes causes the virulence of the 17XL line to be reduced to the same level as the nonlethal 17X line, suggesting that *Py*EBL is a critical virulence determinant in the 17XL line. The parasitemia of mice infected with 17X-CtoR increased significantly compared with those infected with parental 17X and control 17X-CtoC lines during the acute phase of infection on days 4 to 5 ($P < 0.001$). However, the parasitemia did not reach the level observed for the lethal 17XL line, and it reduced to the same level observed for the 17X and 17X-CtoC lines by day 9 (Fig. 5*B*). No parasites were detectable by microscopy at day 17 (not shown). This suggests that the 17X-CtoR line is able to invade a greater repertoire of erythrocyte types than 17X but is unable to invade as many types as the 17XL line. This reduced capacity to invade multiple erythrocyte types compared with the 17XL line results in a nonlethal infection, in which all mice survive (Fig. 5*C*). Thus, displacement of the EBL from microneme was not sufficient to make this line fully lethal, suggesting the existence of other determinant(s).

Discussion

The results of this study indicate that replacement of Cys to Arg at the second Cys position of *Py*EBL region 6 is the major

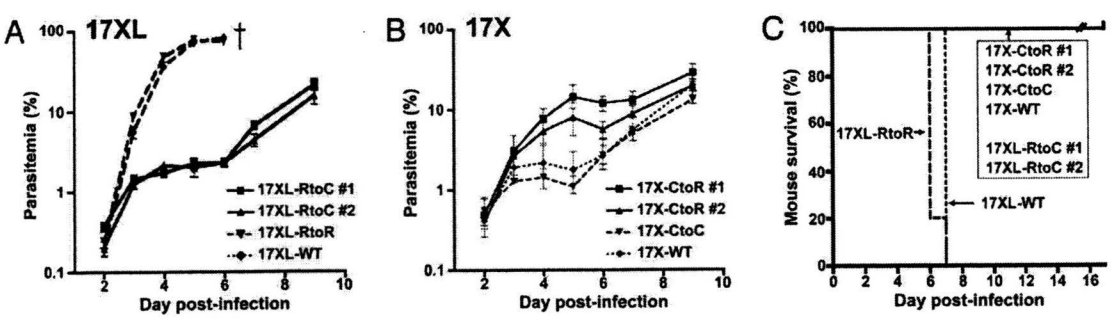

Fig. 5. Effect of the alteration of *pyebl* gene loci on the course of infection and parasite virulence in mice. Mice were i.v. inoculated with 1×10^6 parasitized erythrocytes from WT or transgenic parasite lines. (*A*) Parasitemia of 17XL-RtoC was dramatically reduced to the same level as that of the nonlethal 17X line. (*B*) Parasitemia of 17X-CtoR was significantly higher than parental 17X and control 17X-CtoC on days 4 and 5 ($P < 0.001$), the acute phase of infection; however, the pattern observed is intermediate between the lethal 17XL and nonlethal 17X lines. Parasitemias are plotted using the geometric mean and SD of log-transformed data from groups of 5 mice. (*C*) All mice infected with 17XL-RtoC survived, whereas all mice infected with parental 17XL and control 17XL-RtoR lines died by day 7. All mice infected with 17X, 17X-CtoC, and 17X-CtoR survived.

determinant of the difference between lethal and nonlethal lines of *P. y. yoelii* parasites. This substitution alters the intracellular organelle localization of *Py*EBL from the micronemes to the dense granules and alters the erythrocyte-type invasion preference, course of infection, and parasite virulence in the host.

The crystal structure of region 6 of *P. falciparum* EBA-175 indicates that the second Cys residue forms a disulfide bridge with the fourth Cys residue in this region. Arg substitution of the second Cys residue in the *P. yoelii* 17XL line abolishes this disulfide bridge and thus likely destroys the region 6 structure, which is critical for the trafficking of the protein to the micronemes. It is possible that an incorrectly folded region 6 would not allow the protein to be properly recognized by an (as yet uncharacterized) partner molecule responsible for the trafficking of the EBL protein to the micronemes (9). The mechanism involved in the trafficking of the mutated protein to the dense granules remains unresolved.

Using genetic modification, we have demonstrated that when *Py*EBL is trafficked to the microneme in the 17XL line genetic background, the erythrocyte-type invasion preference and the course of infection are comparable to those of the nonlethal 17X line. This indicates that the substitution of Cys to Arg is a major determinant of the lethal phenotype of the 17XL line. However, when *Py*EBL was not trafficked to the microneme in parasites with the 17X line genetic background, the course of infection was intermediate between the 2 parental lines, suggesting that although *Py*EBL is a critical determinant, other factor(s) are also involved in the lethal phenotype of the 17XL line. In *P. falciparum*, the expression of EBL seems to be co-operationally regulated with another *Plasmodium* ligand encoded by the *rbl (reticulocyte-binding-like)* multigene family that is composed of 6 members in *P. falciparum* and at least 14 members in *P. yoelii* (14–16); thus, the *P. yoelii rbl* protein, Py235, is a potential candidate for such factor(s). Consistent with this hypothesis is the finding that when Py235 expression was suppressed, the course of infection of the lethal *P. yoelii* YM line was altered from a lethal pattern to an intermediate pattern similar to that observed in the 17X-CtoR line shown in this study (17). On the basis of these observations, we propose that *Py*EBL may preferentially recognize reticulocytes and that the removal of *Py*EBL from the micronemes may result in free space within this organelle that may subsequently be filled with other ligand(s), possibly Py235, which consequently enables the parasite to invade a variety of erythrocyte types. Because different Py235 proteins may have different receptor specificities, parasite invasion preference and the subsequent course of infection may vary, depending on the Py235 member that fills the free space in the micronemes created by the absence of *Py*EBL. Such a switching mechanism for an erythrocyte invasion pathway has been previously proposed for *P. falciparum* (18).

A Linkage Group Selection analysis conducted by Pattaradilokrat et al. (19) identified a chromosomal region that included the *ebl* gene locus as a major determinant in the multiplication rate differences between the lethal *P. y. yoelii* YM line and a nonlethal 33X line, supporting the role of the EBL protein in controlling virulence phenotypes. Consistent with our findings that another genetic factor may be involved, they also identified a further genomic region on *P. yoelii* chromosome 5 or 6 that showed weak association with multiplication rate.

Because *Py*EBL localized in the dense granules is potentially nonfunctional, we attempted to disrupt the *pyebl* gene locus in both the 17X and 17XL lines (Fig. S7). However, repeated attempts failed to achieve this, despite the successful genomic integration of the control plasmid. This indicates that *Py*EBL is essential for parasite survival, even when it is not trafficked to the microneme. Two possible explanations for this may be that (*i*) an undetectable amount of *Py*EBL may still localize in the micronemes and remain functional, or (*ii*) *Py*EBL is functional during erythrocyte invasion (or for another unknown critical role during the life cycle), even when localized in the dense granules. Although a subgroup of the dense granules, known as exonemes, were recently reported to secrete their contents immediately before schizont rupture (20), we found that *Py*EBL was not detected on the surface of released individual merozoites of 17XL parasites (Fig. S8). Thus, the identity of the *Py*EBL-containing dense granules and the timing of *Py*EBL secretion from them, if at all, in the 17XL line remain undetermined.

In summary, we have found that a single nucleotide substitution altered the intracellular localization of the malaria parasite ligand *Py*EBL, which in turn altered erythrocyte invasion preference, course of infection, and parasite virulence. The virulence-mediating mechanism described in this report furthers our understanding of parasite–host interactions and has important implications for malaria vaccine design, especially those based on *Pv*DBP for *P. vivax* malaria.

Materials and Methods

Rodent Malaria Parasites. *Plasmodium yoelii* 17X, 17XL, and YM lines were maintained in BALB/c mice (Charles River Japan). The *P. yoelii* YM line was a kind gift from David Walliker of Edinburgh University.

DNA and RNA Isolation. Parasite genomic DNA (gDNA) was isolated from parasite-infected mouse blood using DNAzol BD reagent (Invitrogen). Parasite-infected blood was passed through a single CF11 cellulose column to remove leukocytes, and a schizont-enriched fraction was collected by differential centrifugation on a 50% Percoll solution (GE Healthcare). Total RNA was isolated from the schizont-enriched fraction using RNeasy Mini Kit (Qiagen). cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) with random hexamer after DNase treatment.

PCR Amplification and Sequencing of *ebl* Genes. *ebl* genes were PCR-amplified from gDNA using KOD Plus DNA polymerase (Toyobo), with specific primers for each *ebl* gene designed using the *P. yoelii* genome database (The Institute for Genomic Research) and the *P. chabaudi* and *P. berghei* genome databases (The Sanger Centre). *ebl* sequences were determined by direct sequencing using an ABI PRISM 310 genetic analyzer (Applied Biosystems) from PCR-amplified products. Sequences were aligned using CLUSTALW implemented in MacVector (version 9.0; Accelrys).

Southern Blot Analysis. Five micrograms of *P. yoelii* gDNA were digested with EcoRI, EcoRV, ClaI, BanI, and NspI, and with BanI and HpaI with appropriate buffer, overnight. Digested gDNA was subjected to electrophoresis on 0.8% agarose gels, followed by alkaline transfer onto a Hybond-*n* + PVDF membrane (GE Healthcare). Probes were first PCR-amplified with 5′-TAAATCTAAATGGGATACAT-3′ and 5′-AGTTGGATTGATAGTTACAGATTC-3′ primers for the *pyebl* region, cloned into pGEM-T Easy plasmid (Promega), digested from the plasmid, and then hybridized onto membranes. Probes were labeled with the AlkPhos Direct kit (GE Healthcare), and a chemiluminescent signal developed with CDP-*star* reagent (GE Healthcare) was recorded on RX-U film (Fujifilm).

Recombinant Proteins. Expression plasmids were constructed on the basis of the pEU-E01-G(TEV)-N2 vector (21) by inserting PCR products amplified from *P. yoelii* 17X gDNA using KOD Plus DNA polymerase with the following primers: 5′-gagaCTCGAGGTTAATTTATTAAAAAGAACATATGAATCTTTCC-3′ and 5′-tctcGGATCCCTATGAATAGCTCTCTTTTTGAAAAC-3′ for *Py*EBL regions 1 to 6 (R1–6; amino acid positions 28–787), 5′-gagaCTCGAGGTTAATTTATTAAAAAGAACATATGAATCTTTCC-3′ and 5′-tctcGGATCCCTACAAATTATTATTAATAGGAGTATTACTGGG-3′ for regions 1 to 2 (R1–2; 28–436), 5′-gagaCTCGAGGAAAAAAATGGAAATGTAAATTACAAAG-3′ and 5′-tctcGGATCCCTACAAATTATTATTAATAGGAGTATTACTGGG-3′ for region 2 (R2; 113–436), 5′-gagaCTCGAGTCTTCTGTTAAACCCAGTAATAC-3′ and 5′-tctcGGATCCCTATACATTTTCGTTGGCTAGC-3′ for regions 3 to 5 (R3–5; 423–716), and 5′-gagagagaCTCGAGGACCCTAAACATGTATGTGTTGATAC-3′ and 5′-gagagagaGGATCCTCATCCCATAAAGCTGGAAGAACTACAG-3′ for the 19-kDa region of the merozoite surface protein 1, *Py*MSP1 (*Py*MSP1–19; 1658–1757). The stop codon is shown in bold letters, and XhoI and BamHI restriction sites are underlined. GST-fused *Py*EBL or *Py*MSP1–19 recombinant proteins were expressed using the wheat germ cell-free protein synthesis system (Protemist DT; CellFree Sciences). Recombinant proteins were captured by a glutathione

column, washed, and eluted with glutathione elution buffer. Protein synthesis was confirmed by SDS-PAGE and Coomassie Brilliant Blue protein staining. Recombinant *Py*EBL R1–6 and R3–5 and *Py*MSP1–19 were used to produce antibodies, and *Py*EBL R1–2 and R2 were used for Western blot analysis.

Antibodies. To produce mouse anti-*Py*EBL and anti-*Py*MSP1 sera, female BALB/c mice were i.p. immunized 5 times with recombinant *Py*EBL R1–6 or 3 times with recombinant *Py*MSP1–19 emulsified with Freund's adjuvant, and killed for serum collection. To produce rabbit anti-*Py*EBL R3–5 serum, a female Japanese white rabbit was s.c. immunized 3 times with recombinant *Py*EBL R3–5 emulsified with Freund's adjuvant. To produce mouse anti-*Py*EBL monoclonal antibodies, the spleen was removed from a mouse immunized with recombinant *Py*EBL R1–6, and spleen cells were fused with a mouse myeloma cell line derived from a BALB/c mouse by the conventional polyethylene glycol method. Supernatants of cultured hybridoma colonies were tested with recombinant *Py*EBL R1–6 by ELISA and on *P. yoelii* 17X blood smears by indirect immunofluorescent assay. Positive hybridoma colonies were selected and cloned by 2 rounds of limiting dilution. The epitope region of each monoclonal antibody was tested by Western blot with a panel of recombinant *Py*EBL proteins. Anti-AMA1 rabbit serum was a gift from Carole Long of the National Institutes of Health.

Immunofluorescence Microscopy. *P. yoelii*–infected mouse erythrocytes were smeared onto glass slides, air dried, and stored at −80 °C without fixation. Slides were thawed, acetone-fixed, preincubated with PBS containing 5% nonfat milk at 37 °C for 30 min, incubated with mouse anti-*Py*EBL and rabbit anti-AMA1 sera at room temperature for 1 h, and then incubated with FITC-conjugated goat anti-(mouse IgG and IgM) antibody (Biosource International) and Alexa-546–conjugated goat antirabbit IgG antibody (Molecular Probes) at 37 °C for 30 min. Parasite nuclei were stained with DAPI. Differential interference contrast (DIC) and fluorescent images were obtained using a fluorescence microscope (BX50; Olympus) with a CCD digital camera (DC500; Leica) and processed using Adobe Photoshop CS (version 8.0; Adobe Systems).

Immunoelectron Microscopy. *P. yoelii*–infected mouse blood was fixed in 1% paraformaldehyde–0.1% glutaraldehyde in Hepes-buffered saline and embedded in LR white resin (Polysciences). Sections were blocked for 30 min in PBS–milk–Tween 20, incubated overnight at 4 °C in PBS–milk–Tween 20 containing mouse anti-*Py*EBL R1–6 serum, and then incubated for 1 h in PBS–milk–Tween 20 containing goat antimouse IgG conjugated with gold particles (10 nm diameter; Jansen). Sections were stained with 2% uranyl acetate in 50% methanol and examined by electron microscopy (JEM-1230; JEOL).

Genetic Modification of the *pyebl* Gene Locus. Two basic plasmids, pPbDT3U-B12 and pHDEF1-mh-R12, were constructed. A DNA fragment encoding cyan fluorescent protein was PCR-amplified from pECFP-C1 plasmid (Stratagene) using KOD Plus DNA polymerase with primers 5′-agcGCTAGCGTGAG-CAAGGGCGAG-3′ (NheI site is underlined) and 5′-gacGTCGACGGATCCTCTA-GACTTGTACAGCTCGTCC-3′ (SalI and XbaI sites are underlined, and BamHI site is shown in bold) and ligated into the pGEM-T Easy plasmid. The insert was then digested with NheI and SalI, purified, and ligated into pRGDT-B12 (22) using the NheI and SalI sites, yielding pRCDT-B12. pRCDT-B12 was digested with ClaI and XbaI and filled with an oligonucleotide linker comprising cgatCTCGAGCCCGGGt and ctagaCCCGGGCTCGAGat to generate XhoI (underlined) and SmaI (bold) sites to yield pPbDT3U-B12. pHDEF1-mh (23) was digested with SmaI and ApaI to remove the 3′ untranslated region of histidine-rich protein 2, the ApaI cohesive end was blunted, and a Gateway gene conversion cassette C1 (Invitrogen) was inserted. The XhoI site was destroyed by XhoI digestion, filled in using KOD Plus DNA polymerase, and self-ligated to yield pHDEF1-mh-R12.

To modify the *pyebl* gene locus, a DNA fragment encoding *Py*EBL region 6 to the stop codon was PCR-amplified from gDNA of the *P. yoelii* 17XL line with primers 5′-gCCATGGGAACATAGAGACATTAAAAAAAGC-3′ and 5′-gCTCGAGATAAAAATCTACAGGTATATATTC-3′ (NcoI and XhoI sites are underlined) and cloned into pGEM-T Easy plasmids. The insert was ligated into the NcoI and XhoI sites of pPbDT3U-B12 to yield pR6Cyt-B12. DNA fragments encoding *Py*EBL region 3 to the stop codon were PCR-amplified from cDNA of the *P. yoelii* 17X and 17XL lines with primers 5′-atCTTCTGTTA-AACCCAGTAATAC-3′ and 5′-ccAGATCTTTAATAAAAATCTACAGG-TATATATTC-3′ (BglII site is underlined). PCR products were then ligated into the SmaI site of pR6Cyt-B12, yielding pR6Cyt+R3Cyt(X)-B12 and pR6Cyt+R3Cyt(XL)-B12, respectively. pR6Cyt+R3Cyt(X)-B12 and pR6Cyt+R3Cyt(XL)-B12 were subjected to a BP recombination reaction with the donor vector pDONR221 (Invitrogen) to produce the corresponding entry plasmids pENT.R6Cyt+R3Cyt(X) and pENT.R6Cyt+R3Cyt(XL). These entry plasmids were subjected to a LR recombination reaction (Invitrogen), according to the manufacturer's instructions, with pHDEF-1-mh-R12 to yield replacement constructs pYEBL-R6Cyt+R3Cyt(X) and pYEBL-R6Cyt+R3Cyt(XL), respectively.

P. yoelii schizont-enriched fraction was collected by differential centrifugation on 50% HistoDenz in PBS, and 20 μg of XhoI-digested transfection constructs were electroporated to 5×10^7 of enriched schizonts using the Nucleofector device (Amaxa) with human T cell solution under program U-33 (24). Transfected parasites were i.v. injected into 8-week-old BALB/c female mice, which were treated by i.p. injection with 1 mg/kg of pyrimethamine daily. Before inoculation of 17X line parasites, mice were treated with phenylhydrazine to increase the reticulocyte population in the blood. Drug-resistant parasites were cloned by limiting dilution. Integration of the transfection constructs was confirmed by PCR amplification with a unique set of primers for the modified *pyebl* gene locus, followed by sequencing and Southern blot analysis.

Course of Infection. To assess the course of infection of transgenic and WT parasite lines, 1×10^6 parasitized erythrocytes were injected i.v. into 8-week-old female BALB/c mice. Thin blood smears were made daily, stained with Giemsa's solution, and parasitemias were recorded. Mouse survival was evaluated by the Kaplan-Meier method. Parasitemias of each group were compared by 1-way ANOVA and Tukey's posttest, implemented in Prism 4.0 (GraphPad Software).

Selectivity Index. To compare erythrocyte preference between transgenic and WT *P. yoelii* parasite lines, a SI was calculated as follows: Multiple-infected erythrocytes divided by the expected number of multiple-infected erythrocytes, which was calculated from the number of infected erythrocytes and parasitemia (13). When the preferred erythrocyte type is limited, the observed number of multiple-infected erythrocytes increases. More than 200 parasitized erythrocytes were examined on Giemsa-stained thin blood smears collected on postinoculation day 3. The SI of each group was compared by 1-way ANOVA and Tukey's posttest, implemented in Prism 4.0.

For additional information see *SI Materials and Methods.*

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Expert Opinion

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Wheat germ cell-free technology for accelerating the malaria vaccine research

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Background: Malaria causes about 300 million illnesses and 1 million deaths annually. The likeliest scenario is the aggravation of this disease due to the re-emergence of drug-resistant parasites and insecticide-resistant mosquitoes. One of the promising solutions to this disease are vaccines. However, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts. Even the efficacy of RTS,S, the most advanced vaccine candidate in the pipeline of malaria vaccine development, is only around 50%. *Objective*: Against this backdrop, there is an urgency to rapidly enrich the pipeline of vaccine development with novel vaccine candidates that can be discovered by synthesizing and screening a multitude of malaria proteins. *Methods*: However, to achieve this objective, we require optimal technologies for high-throughput synthesis of quality malaria proteins. Among the various protein synthesis systems, the wheat germ cell-free protein synthesis system is advantageous and successful to this end. *Results/conclusion*: The wheat germ cell-free protein synthesis system is optimal for accelerating the decoding of malaria genome and hence characterization of malaria proteins and discovery of malaria vaccine candidates.

Keywords: malaria, *Plasmodium falciparum*, post-genome, vaccine candidate discovery, wheat germ cell-free protein synthesis

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1. Introduction

Malaria is a serious infectious disease caused by protozoan parasites of the genus *Plasmodium*. The most deadly form of the disease is caused by the inoculation of *Plasmodium falciparum* by infected mosquito bites. This disease causes deaths in millions of people annually and illness in about 300 million people and also puts the health of 3.3 billion people at risk [1]. The complex life cycle of the parasite starts when sporozoites are inoculated during the bite of female *Anopheles* sp. mosquito infected with *Plasmodium*. The sporozoites in the peripheral blood circulation then invade the liver and develop into schizonts containing thousands of merozoites. The schizonts in the hepatocytes then rupture and release into the blood stream the merozoites capable of invading red blood cells. This initiates an intraerythrocytic cycle which comprises the development of ring, trophozoite and schizont stage parasites, then rupture of schizonts, and release of free merozoites for reinvasion into the erythrocytes. This developmental cycle takes about 48 h. These asexual erythrocytic-stage parasites are responsible for the clinical manifestations and pathology of malaria, the most serious illnesses being anemia and cerebral malaria [2].

Despite the parasite's complex life cycle, high level of antigenic diversity and mechanism of immune evasion, naturally acquired immunity to malaria indeed develop after repeated exposure over a period of several years. Even though this natural protective immunity possibly never leads to sterile immunity as suggested by

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the fact that parasites can still be found in the circulation of adults living in high transmission settings, this immunity still confers protection against symptomatic disease, high-density parasitemia and death [3,4]. The naturally acquired immunity seems to predominantly target blood-stage parasites [5]. The importance of antibodies in passive immunization has been supported by the transfer of immunoglobulin conferring protection from immune individuals to non-immune individuals [6,7]. Furthermore, immunity against severe, life-threatening malaria is apparent after a few disease episodes, suggesting that the target antigens have limited diversity in parasite populations [8]. The immune effector mechanisms are poorly understood; however, it is widely understood that antibodies may have a role in preventing merozoite invasion, clearance of infected erythrocytes, prevention of adhesion and sequestration of parasitized infected erythrocytes in the vasculature, and prevention of schizont rupture. Functionally, antibodies could prevent erythrocyte invasion by opsonizing merozoites and facilitating phagocytosis by macrophages, activating complement-mediated damage of merozoites [3]. It is becoming increasingly clear, however, that cell-mediated immunity may also be a critical component of naturally acquired immunity to malaria [9]. In addition to the naturally acquired immunities mentioned above, there are encouraging experimental evidences that the sterile, long-lasting protective immunity was induced, after vaccination with radiation-attenuated sporozoites, in mice [10] and humans [11], or after monthly inoculation of sporozoites through mosquito bites to human volunteers under a prophylactic regimen of chloroquine which prevent only the growth of blood-stage parasites [12]. The above-mentioned evidences strongly support the strategy of malaria control through vaccination that elicits protective immunity.

2. Current situation on malaria vaccine development

Malaria is a leading cause of mortality and morbidity. The efficacy of malaria control through current interventions that use drugs and insecticides may not be sustained too long because they rely on too few compounds [4]. In fact, the disease is re-emerging mainly due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes [13]. Very recently, there is a disturbing report on the emergence of parasite's resistance even to artemisinin [14]. Therefore, we are now forced to endeavor to develop malaria vaccines as one of the essential components for the malaria eradication [15]. We should also keep in mind the fact that, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts [16].

Malaria vaccines are classically categorized into three groups based on the stage of the parasite in which the vaccine works. In other words, the parasite presents different proteins as antigens in different stages of life cycle. Pre-erythrocytic vaccines, either based on subunit vaccines or live attenuated parasite vaccines, have been designed to prevent infection and development of diseases. Protection is, to some extent, mediated by antibodies that prevent sporozoite invasion into hepatocytes [17]. Currently, the RTS,S, a pre-erythrocytic vaccine candidate that uses a fragment of *P. falciparum* circumsporozoite protein fused to the HBV surface antigen [18], is undoubtedly the most advanced and promising vaccine candidate based on a number of clinical trials [19-22]. However, the efficacy reported in these trials was around only 50%, suggesting that there is an urgent need to develop new adjuvant systems, test new boosting strategies and enrich the pipeline of vaccine development with additional promising vaccine candidates.

Asexual blood-stage vaccines are aimed at reducing parasite multiplication and growth in order to protect against clinical symptoms and particularly severe disease, and not infection. They are designed to induce antibody responses against the asexual blood stage of the parasites. Asexual blood stage parasite development is a repeating cycle of the release of new merozoites from schizonts and invasion of merozoites into naive erythrocytes. The ability of the merozoite to specifically attach to and invade erythrocytes is essential for blood stage development; for example, *Plasmodium vivax* must bind to the Duffy antigen to invade into reticulocytes [23]. This and other findings have inspired the search for merozoite antigens that elicit antibodies that block parasite invasion of erythrocytes. Immunization with blood-stage antigens, mainly with different merozoite antigens, has been shown to be protective in a number of animal models [24-27].

For example, Combination B, a vaccine combining merozoite surface protein-1 (MSP1) and MSP2 and a part of the ring-infected erythrocyte surface antigen when tested in humans conferred some level of strain specific protective effect. This vaccine showed promising effect in reducing the severity of malaria episodes but the effect is specific only to 3D7 type allele of MSP2, not to FC27 type allele. Because of this, this vaccine subsequently became ineffective in controlling morbidity, due to the selection pressure of this vaccine and subsequent emergence of the parasite with FC27 type allele of MSP2. This fact strongly argues for the development of vaccines comprising multiple components covering all major allelic types [4]. At present, the leading blood-stage vaccine candidates are all merozoite proteins, either located on the surface or within the apical organelles of merozoites. However, none of the merozoite antigens that have been tested in humans, including MSP1, MSP3 and apical merozoite antigen-1 (AMA1), has yet been shown convincingly to confer high levels of protection in clinical trials [28]. Unlike *P. vivax*, *P. falciparum* uses multiple redundant pathways to invade erythrocytes, complicating the effort to develop anti-invasion vaccines against *P. falciparum* [29,30].

Blood stage immunity might also target variant proteins encoded by the parasite multi-gene families (e.g., *var* gene families). PfEMP1, the most famous protein encoded by the *var* gene family, is exported by parasites onto the surface of the

infected erythrocytes (IRBC) and this protein is involved in IRBC adhesion to endothelium and immunoevasion [31]. The best example of this type of immunity has been demonstrated during pregnancy. In pregnant women, parasites emerge that express IRBC surface proteins, allowing these IRBC to bind chondroitin sulfate A (CSA) and sequester in the placenta [32]. First-time mothers lack antibodies specific for the IRBC surface proteins of these parasites and are highly susceptible to infection and disease. Women become resistant over successive pregnancies as they acquire antibodies that block IRBC binding to CSA [33]. Placental parasites express distinct genes and proteins, including an IRBC variant surface protein called VAR2CSA that is required for adhesion to CSA. A program to develop a vaccine based on VAR2CSA is underway [17,34-38]. This fact strongly argues for the development of vaccines comprising conserved epitopes covering all major variant genes [4].

Recently, there has been a renewed call for the 'eradication' of malaria [39]. An effective vaccine will probably be needed to achieve eradication, in addition to other existing drugs and insecticides-based measures. To achieve malaria eradication, the emphasis is on approaches that reduce not only clinical illness in humans but also parasite transmission from humans to mosquitos. Therefore, vaccines that confer sterile immunity and/or transmission-reducing activity would be preferred. The role of asexual blood-stage vaccine as part of this malaria eradication strategy has been questioned, as this vaccine is generally believed to reduce parasitemia and prevent clinical illness but not affect transmission. However, it is also possible that, in addition to preventing clinical illness, an effective blood-stage vaccine may also contribute to the reduction in the efficiency of the transmission by interrupting the blood-stage life cycle in the human body [28].

Transmission-blocking vaccines are aimed at blocking malaria transmission by interrupting the parasite life cycle in the mosquito. These vaccines elicit antibodies against antigens that are expressed by the sexual stage of the parasite and, thus, stop their subsequent development in the mosquito midgut [40]. The target antigens of this vaccine were shown to be sexual-stage specific surface molecules (e.g., P230 and P48/45) that are involved in the process of fertilization of male and female gametes. Subsequently, other antigens (e.g., P25 and P28) that are uniquely expressed by zygotes and ookinetes in the mosquito midgut were shown to be equally good for induction of transmission-blocking immunity. Antibodies in experimental animals against Pfs25 and Pvs25 have been successful at preventing parasite transmission [41,42] and, therefore, these vaccine antigens, Pfs25 [43] and Pvs25 [44], were tried out in Phase I clinical trials. Such vaccines will not provide any immediate direct benefit to the vaccinated individual, but their widespread deployment will help reduce transmission of the parasite and thus protect both the vaccinated individuals and their community as a whole [45]. These transmission-blocking vaccines, if used in combination with pre-erythrocytic or erythrocytic vaccines, might also prevent the transmission of mutant parasites and parasites expressing variant alleles that emerge by evading and escaping human protective immune responses. When used in combination with vector control measures, the transmission-blocking vaccine could play a key role in finally breaking the transmission of parasites, leading to eradication of the diseases [46].

An effective malaria vaccine is expected to target several antigens expressed at different stages of parasite [47] and induce protective immune responses equivalent to, or better than, those provided by naturally acquired immunity or immunization with attenuated whole parasite [48]. In order to accelerate the discovery of such vaccine candidates, we need two things. One is the optimal system for high-throughput synthesis of malaria proteins and the other is the efficient post-genomic high-throughput approaches for screening and selecting potential vaccine candidates from these synthesized proteins.

3. How the malaria genome data can be exploited profitably for malaria vaccine candidate discovery

Decades of research in the pre-genomic era have identified only a handful of vaccine candidates [4]. With the recent completion of the genome projects of human malaria parasites, *P. falciparum* [48], *P. vivax* [49], zoonotic primate malaria, *Plasmodium knowlesi* [50] and rodent malaria parasite, *Plasmodium yoelii* [51], we are now in the post-genome era. Thousands of novel genes have been annotated, providing new opportunities for vaccine research. In order to successfully identify putative novel vaccine candidates for downstream vaccine research, we now need to thoroughly analyze these genomic data using at least two post-genomic high-throughput approaches. One is the functional approach (otherwise called reverse vaccinology [52]) and the other is the immunoscreening approach.

In the functional approach, several direct and indirect functional criteria are used to objectively select and prioritize putative vaccine candidates for further functional studies [53]. For example, the putative candidates for vaccine research can be selected based on their role in erythrocyte invasion [54], essentiality for the parasite's survival revealed by gene knockout studies [30], putative secretory function predicted based on presence of signal peptides and transmembrane regions, localization on the surface or in the apical organelles of the merozoite, or signatures of immune selection pressure revealed by polymorphisms and diversity studies [55].

In the immunoscreening approach, hundreds or thousands of malaria proteins as antigens are screened, using immunoassays such as enzyme-linked immunosorbent assay and so on, with a large number of human serum samples obtained from non-immune, semi-immune and immune individuals. This profiling of immune responses that develop in humans, after natural or experimental infection, or after vaccination with irradiated or genetically attenuated organisms, will facilitate

identifying immunoreactive and protective antigens [56]. Whatever may be the approach used for the discovery of novel vaccine candidates, we do need to have an optimal recombinant protein synthesis system not only for the synthesis and characterization of these vaccine candidates in the initial stage, but also for the mass production of vaccine antigens for vaccination purpose in the later stage.

The first and the foremost barrier to the rapid discovery of vaccine candidates is the lack of an efficient system to synthesize quality malaria proteins. *Plasmodium falciparum* genes are A/T rich (average A/T content per coding sequence is 76%) and a number of them encode repeated amino-acid motifs [48], and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional *Escherichia coli* cell-based systems [57-59]. Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses [60-62]. There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity, and requirement of preparation of large quantities of antigen for immunization. The above impediments are completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus or Chinese hamster ovary cell. But, fortunately, we learnt that the wheat germ cell-free system could surmount most of the above stumbling blocks in the path of post-genome malaria vaccine candidate discovery [63].

4. Wheat germ cell-free protein synthesis system

Recently, the wheat germ cell-free protein synthesis system was established for practical use in protein production [64] and is now commercially available as simple protein synthesis kits including *in vitro* transcription system and translation system (CFS Co., Ltd, Matsuyama, Japan). The system is especially powerful when used for the production of eukaryotic proteins because of its eukaryotic nature [65,66]. Basically, two wheat germ cell-free protocols were established for practical use [65]. The first one is for small scale synthesis of proteins. It can be used to produce small quantities of proteins from a large number of cDNAs, in parallel, for testing synthesis and solubility of proteins and also for genome-wide biochemical annotation of gene products [67]. The second protocol is for the production of large quantities of proteins [68]. In this protocol, genes of interest are cloned into the pEU wheat germ expression vectors [69,70] and the mRNA is transcribed. Then, the mRNA is translated either using the bilayer or discontinuous batch translation method [65]. The bilayer method can produce hundreds of micrograms of protein. The discontinuous batch translation method can produce around 150 mg of a control protein in a reaction volume of 50 ml in 5 h and gives room for scale up [68]. These two methods have already been acknowledged as advantageous to yield good quality proteins, in the fields of structural and functional genomics of eukaryotes [66,71]. If we could establish a good manufacturing practice facility for production of recombinant proteins for clinical studies such as vaccine research using this system, then this system may be suitable for mass production of vaccine. The fact that both the wheat germ system and the malaria parasite do not have glycosylation machinery is an advantage for expressing malaria proteins without any inadvertent glycosylation in wheat germ system. Taken together, this system is versatile for both small and large scale production of quality malaria proteins.

We found from our initial studies that the wheat germ cell-free system can surmount most of the above impediments and hence accelerate the discovery of malaria vaccine candidates [63]. Initially, in order to test the suitability of wheat germ cell-free system for high-throughput expression of the parasite genes, we tried to express 124 genes encoding asexual blood-stage parasite proteins, selected from the malaria genome database, PlasmoDB (http://plasmodb.org/plasmo/). Seventy-five percent of genes (93 out of 124 genes) yielded soluble protein products. However, the extent of solubility among these 93 soluble proteins was on an average 65% and ranged from 26 to 100% [63]. The average yield of expressed protein estimated for each full size product was 1.9 µg per 150 µl of reaction mixture, an amount sufficient for preliminary antigen discovery studies using hyper-immune serum. We found that there was significant inverse correlation between yield and molecular size of the protein; the greater the size, the lower the protein yield. Because of this reason, it will be difficult to synthesize the full size of high molecular mass vaccine candidates, such as PfEMP1. There was also an inverse correlation between the protein yield and the abundance of low-complexity regions. In addition, solubility was inversely correlated with the isoelectric point (pI) value [63]. Similar observations have been also documented in *E. coli* cell-based expression system [58,59]. Surprisingly, we did not see any correlation among yield and A/T content, pI value or the existence of a transmembrane domain [63]. From these pilot experiments, we found that the wheat germ system can be used successfully for the expression of *P. falciparum* genes.

Later on, from many of our and our collaborator's proof-of-principle studies, we confirmed that wheat germ cell-free protein synthesis system is advantageous when it comes to synthesizing biologically active malaria proteins by directly using the native A/T rich malaria genes without any codon optimization (summarized in **Table 1**). First, the system was able to produce active malaria enzymes, such as *P. falciparum* dihydrofolate reductase-thymidylate synthase [72] and chitinase of *P. vivax* [73]. Localization of pyruvate kinase type-II isozyme was also identified in apicoplast using antibody raised against recombinant protein produced in this system [74]. In fact, all the above mentioned three genes were recalcitrant to expression in other protein expression systems. Second, the wheat germ cell-free system was able to produce a sufficient amount of good quality proteins (an important prerequisite

Table 1. Proof-of-principle studies for the production of biologically active malaria proteins using the wheat germ cell-free expression system*.

Gene ID‡	Gene name	Species§	Results¶	Ref.
Enzymes				
PFD0830w	Bifunctional dihydrofolate reductase-thymidylate synthase	Pf	Enzyme activities (DHFR, TS)	[72]
PVX_087680	Chitinase	Pv	Enzyme activities	[73]
PF10_0363	Pyruvate kinase 2	Pf	Ab (WB, IFA)	[74]
Sporozoite antigens				
PFC0210c	Circumsporozoite protein	Pf	Ab (WB, IFA)	[63]
PFD0215c	P52	Pf	Ab (IFA)	[75]
Merozoite antigens				
PF11_0344	Apical membrane antigen 1	Pf	Ab (WB, IFA)	[63]
PF14_0495	Rhoptry neck protein 2	Pf	Ab (IP, WB, IFA, IEM)	[76]
PY04764	Erythrocyte binding ligand	Py	Ab (WB, IFA, IEM)	[77]
Ookinete antigen				
PF10_0303	Pfs25	Pf	Ab (WB, IFA, TBA)	[63]

*The individual articles may be consulted for further details.
‡Detailed information is available at the PlasmoDB website (http://plasmodb.org/plasmo/).
§*Plasmodium* species, *Pf*; *P. falciparum*, *Pv*; *P. vivax*, *Py*; *P. yoelii*.
¶Results obtained by the contribution of recombinant proteins synthesized by the wheat germ cell-free system.
Ab: Antibody; DHFR: Dihydrofolate reductase; IEM: Immunoelectron microscopy; IFA: Immunofluorescence microscopy; IP: Immunoprecipitation; TBA: Transmission-blocking activity; TS: Thymidylate synthase; WB: Western blot.

for the malaria vaccine candidate discovery), such as a repeat-rich molecule, circumsporozoite protein [63], a cysteine-rich molecule, P52 (one of the target molecules for the generation of genetically attenuated live-sporozoite vaccine) [75], RON2 (merozoite rhoptry neck protein involved in the erythrocyte invasion process) [76], PyEBL (orthologue of EBA175 in a rodent malaria parasite, *P. yoelii*, that is responsible for the merozoite invasion into erythrocyte) [77] and cysteine-rich Pfs25 (a promising transmission-blocking vaccine candidate) [63]. AMA1 requires synthetic codon optimized gene in order to be expressed in *E. coli* cell-based system and the synthesized proteins also need a series of labor-intensive and technically complex refolding processes, because of its cysteine-richness, to obtain quality proteins [78]. Even this recalcitrant AMA1 could be expressed easily without any codon optimization in the wheat germ cell-free system [63].

Recently, we attempted to express 567 of *P. falciparum* cDNA clones selected from sporozoite, merozoite and gametocyte stages in a high-throughput format by the wheat germ cell-free system. Out of 567 genes, 478 (i.e., around 84%) yielded soluble protein products (unpublished). Our biochemical, immunocytochemical and biological analyses have revealed that the recombinant malaria proteins synthesized by this system are of high quality and, therefore, amenable for vaccine candidate assessment.

5. Expert opinion

In order to establish high-throughput genome-wide functional or immunoscreening methods for malaria vaccine candidate discovery, the choice of the recombinant protein synthesis method is the most crucial factor. There were many leading trials to achieve genome-wide expression of *P. falciparum* genes using the conventional *E. coli*-based protein synthesis system. Aguiar *et al.*, using *E. coli* cells, were able to express only 39 out of 292 malaria genes cloned in GST-fusion vector [57]. Mehlin *et al.* carried out an even more challenging trial in which 1000 genes encoding relatively small (< 450 amino acids) malaria cytosolic proteins were expressed in *E. coli* [58]. In this study, only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 – 406 mg of protein per liter of culture medium. Vedadi *et al.* used another approach that used an engineered *E. coli* strain with genetically supplemented tRNAs that allows reading of high number of A/U codons in malaria mRNA [59]. They found marginal improvement in protein solubility, that is, 38 (20.9%) out of 182 proteins tested were soluble. Mu *et al.* [55] and Doolan *et al.* [56] attempted to express malaria recombinant proteins using an *E. coli* cell-free expression system in order to find potential vaccine candidates. Mu *et al.* [55] could express around 60% of the malaria genes (65 out of 108 genes) that are considered to be under immune selection pressure. They verified the expression of proteins via western blot using antibodies to the histidine tags incorporated into the C-terminus of the expressed proteins [55]. Recently, Doolan *et al.* reported the construction of the *P. falciparum* protein microarrays using *E. coli* cell-free protein synthesis system for high-throughput immunoscreening for vaccine candidate discovery [56]. In their study, they obtained > 90% efficiency in their effort to express 250

P. falciparum genes using *E. coli* cell-free *in vitro* transcription and translation reactions. And they have attributed their high success rate in this system, at least in part, to the fact that the system is supplemented with rare transfer RNAs to help translate A/T-rich genes and that the proteins are printed directly without purification on to microarray slides. The above experiments showed better productivity and throughput only in the *E. coli* cell-free system and not in *E. coli* cell-based system. Nonetheless, because the results based on the *E. coli* cell-free system study [55,56] were obtained using a relatively small number of *P. falciparum* genes, they do not truly represent the capacity of *E. coli* cell-free system for genome-wide *P. falciparum* gene expression.

It has been suggested that the eukaryotic translation and folding machinery have been optimized through evolution to facilitate co-translational domain folding of a multi-domain protein. In fact, the rate of peptide growth on ribosomes differs considerably between eukaryotes and bacteria, being five to ten times slower in eukaryotes. This slow rate of peptide growth in eukaryotic protein expression system contributes greatly towards correct conformational folding of a multi-domain protein or its epitope and their solubility [79,80]. Although the *E. coli* system is known to support folding of prokaryotic and small eukaryotic proteins, because of its prokaryotic nature, the proteins with multiple domains that are commonly found in eukaryotes such as malaria parasite proteins, when expressed either cell free or cell-based *E. coli* system, tend to fold incorrectly, resulting in the formation of inclusion bodies [79]. After all, the solubility and the functionality of the correctly folded malaria proteins and/or their epitopes are indispensable and critical factors that directly affect the success of our genome-wide search for potential vaccine antigens. Therefore, from this point of view, the eukaryotic-based system is greatly advantageous over the *E. coli*-based system.

Other cell-free systems derived from eukaryotic sources, including rabbit reticulocytes, yeast cells, tumor cells and insect cell lines, have also been widely used to produce eukaryotic multidomain proteins in active forms, but all suffer from low productivity [65]. One of the most convenient and promising eukaryotic cell-free translation systems for achieving high productivity and high solubility of proteins is conceivably the one based on wheat germ embryos [65].

Recently, Goshima *et al.* have again proved that wheat germ cell-free system is more advantageous than *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In their study, they have successfully expressed human proteins on a whole-proteome-scale [66]. In their study, initially, on a trial basis, they expressed 26 cDNA clones for soluble protein (molecular mass; MM 10.4 – 101.3 kDa) and 24 clones for membrane protein (MM 7.0 – 80.1 kDa) in various expression systems, such as wheat germ cell-free system, *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In this expression study, they found that the wheat germ cell-free system is the best of all the methods for achieving high-throughput, high-quality and high-productivity synthesis of recombinant proteins, and ease of handling. Moreover, of the 75 phosphatases expressed by them in wheat germ cell-free system, 58 (77%) showed biological activity and several cytokines containing disulfide bonds were produced in active forms. They also expressed 13,277 human proteins by this system. The success rate of recombinant protein synthesis was almost 100%. Although the protein-synthesis system exhibits nearly perfect efficacy, the yields of the proteins were variable [66].

In order to discover novel vaccine candidates, the malaria genome has to cross a lot of barriers, such as protein synthesis, selection of protein purification tags, characterization of protein molecules, selection of *in vitro* functional assay systems (i.e., growth inhibition assay or antibody-dependent cellular inhibition assay) and selection of adjuvant. From our experience of expressing around 500 malaria genes using wheat germ cell-free system (unpublished), we learnt that the recombinant malaria proteins synthesized are of high quality and, therefore, amenable for vaccine candidate assessment. Therefore, we indeed believe that this system is a key tool to overcome the first and the foremost barrier, that is, decoding of the malaria genome. From a malaria vaccine perspective, wheat germ system will facilitate production of malaria proteins on a whole-proteome-scale and construction of microarrays of malaria proteins. These microarrays will be an indispensable tool for profiling immune responses developed in non-immune, semi-immune and immune individuals, and identifying immunoreactive antigens that correlate with protection. In fact, we are now focusing on the establishment of microarray of malaria proteins. No doubt, production of malaria proteins on a whole-proteome-scale using wheat germ cell-free protein synthesis system is achievable and microarray of malaria proteins will be soon available for facilitating post-genomic high-throughput identification of novel vaccine candidates.

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Malaria Ookinete Surface Protein-Based Vaccination via the Intranasal Route Completely Blocks Parasite Transmission in both Passive and Active Vaccination Regimens in a Rodent Model of Malaria Infection▽

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Malaria vaccines based on ookinete surface proteins (OSPs) of the malaria parasites block oocyst development in feeding mosquitoes and hence disrupt the parasite life cycle and prevent the disease from being transmitted to other individuals. To investigate whether a noninvasive mucosal vaccination regimen effectively blocks parasite transmission in vivo, *Plasmodium yoelii* Pys25, a homolog of the Pfs25 and Pvs25 OSPs of *Plasmodium falciparum* and *Plasmodium vivax*, respectively, was intranasally (i.n.) administered using a complement-deficient DBA/2 mouse malaria infection model, in which a highly elevated level of oocysts develops in feeding mosquitoes. Vaccinated mice developed a robust antibody response when the vaccine antigen was given together with cholera toxin adjuvant. The induced immune serum was passively transferred to DBA/2 mice 3 days after infection with *P. yoelii* 17XL, and *Anopheles stephensi* mosquitoes were allowed to feed on the infected mice before or after serum transfusion. This passive immunization completely blocked oocyst development; however, immune serum induced by the antigen or adjuvant alone did not have such a profound antiparasite effect. Further, when i.n. vaccinated mice were infected with the parasite and then mosquitoes were allowed to directly feed on the infected mice, complete blockage of transmission was again observed. To our knowledge, this is the first time that mucosal vaccination has been demonstrated to be efficacious for directly preventing parasite transmission from vaccinated animals to mosquitoes, and the results may provide important insight into rational design of nonparenteral vaccines for use against human malaria.

Malaria is one of the most important infectious diseases, and the levels of mortality and morbidity are high, especially among children in developing countries in Africa, Asia, and South America. Implementation of malaria control measures, such as antimalaria drug chemotherapy and insecticide-treated bed nets, has made a significant contribution to reducing the incidence of malaria in many parts of the world. However, these control measures may not be sufficient, and therefore new tools, including vaccines, should be included in a new malaria control campaign for local elimination and final eradiation of malaria from the globe (7). A promising strategy to counteract global malaria endemicity is to develop highly efficacious vaccines, and several promising candidates have been intensively investigated (7, 20); vaccines targeting asexual stages (i.e., sporozoite, hepatic, and erythrocytic stages) are designed to prevent infection and reduce disease severity, while vaccines that target the sexual stage, in which the parasite undergoes sporogonic development in anopheline mosquitoes, prevent vector-mediated transmission of the parasite from person to person (4, 8, 14, 17, 25). Although transmission-blocking vaccines do not directly prevent infection, they reduce parasite infectivity for the vector and consequently lower the mosquito infection rate and the frequency of transmission to humans. In addition, this strategy is believed to be particularly useful for controlling escape of mutants from vaccines designed based on antigens expressed at an asexual stage; therefore, transmission-blocking vaccines are increasingly being considered indispensable components of malaria vaccine strategies and are key components of malaria elimination (10, 11).

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Studies on rodent and human malaria concluded that an effector mechanism that is pivotal for blocking transmission is induction of antigen-specific serum antibodies in a vaccinated host, from which female mosquitoes, when they bite to obtain blood meal, coingest gametocyte pairs together with the induced antibodies (2, 3, 5, 9, 18, 24). The ingested antibodies seem to be stable in the mosquito midgut, at least in the time frame within which the transmitted gametocytes develop into ookinetes.

Parasite antigens expressed later at the postfertilization stage in the mosquito midgut, such as ookinete surface proteins (OSPs), including Pfs25 and Pvs25 from *Plasmodium falciparum* and *Plasmodium vivax*, respectively, are particularly important vaccine targets because they are likely to be concealed immunologically, if not concealed completely, from the mammalian host's immunosurveillance system, which suggests that there is a reduced driving force to produce the antigenic variations often observed for antigens expressed at prefertilization stages (6, 12, 23, 28). In addition, several recent studies indicated that in the malaria life cycle the ookinete-to-oocyst transition stage is one of the most vulnerable stages of parasite development, making the postfertilization stage of sporogonic development an ideal target for antitransmission vaccines.

The vast majority of pathogens invade through mucosal tissues and therefore can be controlled effectively by mucosal vaccines rather than parenteral vaccines. Notwithstanding the great merit of mucosal vaccines, most vaccines in use today are delivered parenterally (subcutaneously [s.c.] or intramuscularly). In spite of many arguments against the concept that vaccines against arthropod vector-borne human pathogens, such as malaria parasites, could be designed based on mucosal delivery, recent studies performed by us and other workers demonstrated that mucosal vaccines could be efficacious for prevention of arthropod-transmitted infections, because mucosal administration of foreign antigens mixed with a potent mucosal adjuvant, such as cholera toxin (CT), can induce strong systemic immunity (2, 3, 13). Here we extended our previous studies to test our hypothesis that the malaria OSPs are sufficiently immunogenic when they are administered by the intranasal (i.n.) route in the presence of a mucosal adjuvant, which should in theory effectively block parasite transmission to feeding mosquitoes when both passive and active vaccination regimens are used.

MATERIALS AND METHODS

Mice, vaccination, and antibody enzyme-linked immunosorbent assay (ELISA). Seven-week-old female DBA/2NCrj (DBA/2) mice were purchased from Japan SLC (Tokyo, Japan). Complement C5-deficient DBA/2 mice were used for live mosquito-feeding experiments, because the highly elevated levels of oocysts that developed in the mosquito midgut were useful for evaluation of transmission-blocking vaccine efficacy (26).

Mice were i.n. vaccinated once a week for 4 weeks with 25 μg of yeast-derived recombinant *Plasmodium yoelii* Pys25 synthesized and purified like Pvs25 as described previously (14) in the absence or presence of 1 μg of CT (Sigma-Aldrich). As a control, a group of mice were vaccinated with 1 μg of CT alone. For passive vaccination experiments, DBA/2 mice were intravenously vaccinated with 0.5 ml of pooled immune sera derived from mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone.

For ELISA of vaccine-induced immune sera, a flat-bottom 96-well microtiter plate (Immulon 4; Dynex Technology Inc., Chantilly, VA) was coated with recombinant Pys25 (0.5 μg/well in bicarbonate buffer, pH 9.6) and blocked with 1% skim milk in Tris-buffered saline containing 0.05% Tween 20. Immune sera serially diluted with the blocking buffer were applied to wells in duplicate (100 μl/well) and incubated for 2 h at 37°C, which was followed by addition of alkaline phosphatase-conjugated anti-mouse antibody for immunoglobulin (Ig) isotype and IgG subclass analysis. The alkaline phosphatase substrate (*p*-nitrophenyl phosphate [Sigma-Aldrich]) was added, and the absorbance at 490 nm was determined with a microplate reader (Bio-Rad Laboratories). The antibody concentration was determined based on known amounts of mouse Igs used as a standard. The statistical significance of differences in antibody concentration or absorbance was determined by Student's *t* test.

Parasite infection, blood feeding experiment, and assay of transmission blocking. For analysis of the parasite-killing effect of i.n. vaccination-induced immune sera, mice were intraperitoneally inoculated with 10^6 peripheral red blood cells that had been infected with *P. yoelii* strain 17XL, and the infected mice were maintained for 3 days until the level of parasitemia reached 9 to 10%, which was determined by microscopic examination of Giemsa-stained thin blood smear preparations. Then approximately 100 *Anopheles stephensi* mosquitoes that had been starved overnight were allowed to obtain a blood meal from the infected mice either before or 1 h after intravenous injection of immune sera that had been prepared from mice 1 week after the last i.n. vaccination with Pys25 plus CT, with Pys25 alone, or with CT alone. Fully engorged mosquitoes were maintained at 24°C for 1 week by giving them water containing 1.5% fructose and 1.5% sucrose. For each experimental group, mosquitoes were dissected, and their midguts were examined with a light microscope to count the number of oocysts.

For analysis of the direct parasite transmission-blocking efficacy of i.n. vaccination, mice vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone were infected as described above with the parasite 1 week after the last vaccination, and then mosquitoes were allowed to feed directly on the infected animals; this was followed by enumeration of the oocysts that developed.

The statistical significance of differences in the numbers of oocysts was determined by the Kruskal-Wallis test or the Wilcoxon-Mann-Whitney U test by using the software JMP (SAS Institute Inc.).

RESULTS

A significant level of specific serum IgG and IgM antibodies (mainly IgG) was induced in DBA/2 mice by i.n. vaccination with Pys25 plus CT (14,147 ± 4,241 μg/ml) but not by i.n. vaccination with Pys25 alone or CT alone (Fig. 1a, upper panel). Oral inoculation of 50 μg of Pys25, however, did not induce an antibody response even in the presence of 10 μg CT (data not shown). IgG1 was found to be the predominant serum IgG subclass, and almost no IgG2a was detected in mice vaccinated with Pys25 plus CT, an indication of the Th2 type of immune response induction (Fig. 1a, lower panel). Low but detectable levels of Pys25-specific serum IgA and IgE were seen in the group vaccinated with Pys25 plus CT but not in the group vaccinated with Pys25 alone or CT alone (Fig. 1b). Similar humoral immune responses were observed when outbred ddy mice were used for the immunization experiments (data not shown).

To evaluate the parasite transmission-blocking effect of the induced immune sera in vivo, *A. stephensi* mosquitoes were allowed to obtain a blood meal from DBA/2 mice that had been infected with *P. yoelii* 17XL before or after passive transfer of the immune sera of mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone as described in Materials and Methods. For all three immunization groups, large numbers of oocysts were observed in the mosquito midgut when the mosquitoes were allowed to feed before the immune sera were transferred (median for CT alone, 346 oocysts; median for Pys25 alone, 302 oocysts; median for Pys25 plus CT, 311 oocysts) (Fig. 2a). In contrast, when the mosquitoes received the blood meal after the immune sera were transferred, oocyst formation was completely blocked in the group vaccinated with Pys25 plus CT, but not in the group vaccinated with

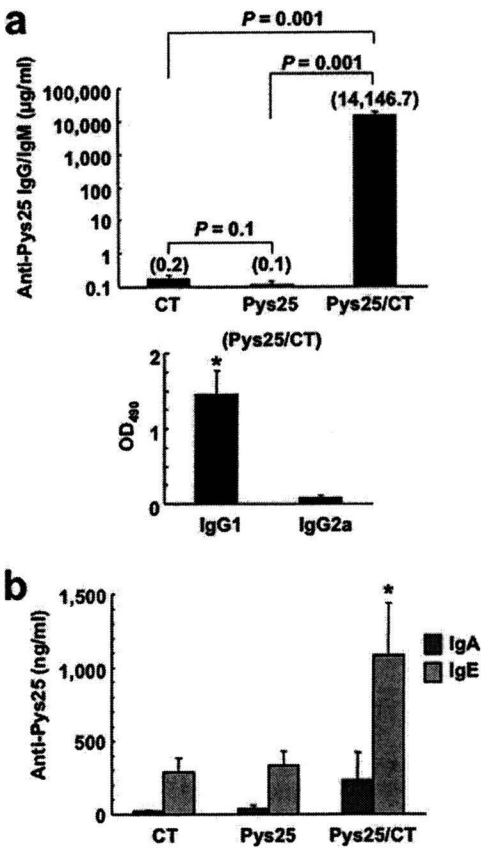

FIG. 1. Antibody responses in mice i.n. vaccinated with recombinant Pys25. DBA/2 mice (three or four mice/group) were vaccinated i.n. with a mixture of Pys25 (25 μg) and CT (1 μg), with Pys25 alone (25 μg), or with CT alone (1 μg) once a week for 4 weeks, and 1 week after the final vaccination serum antibody titers were analyzed by ELISA. (a) (Upper panel) Pys25-specific serum IgG and IgM (predominantly IgG) levels expressed as mean antibody concentrations in the serum. The error bars indicate standard deviations. (Lower panel) Immune sera (1/128,000) from mice vaccinated with Pys25 plus CT were also analyzed for the presence of IgG1 and IgG2a, and the results were expressed as mean optical densities at 490 nm (OD_{490}). The error bars indicate standard deviations. *, $P = 0.002$ for comparison of IgG1 and IgG2a. (b) Pys25-specific serum IgA and IgE levels expressed as mean antibody concentrations in serum (ng/ml). The error bars indicate standard deviations. *, $P < 0.01$ for a comparison of Pys25 plus CT and CT or Pys25. No significant differences between groups were observed for serum IgA. Statistical significance was determined by Student's t test.

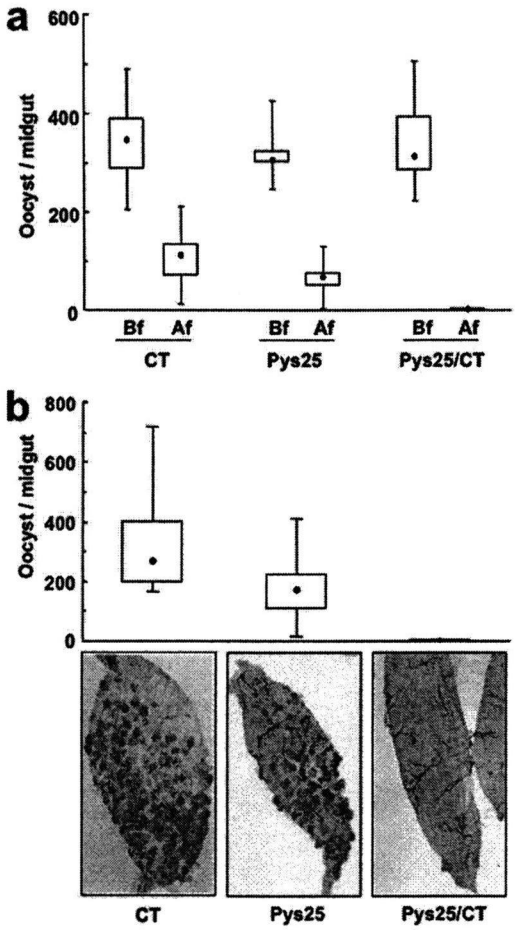

FIG. 2. Transmission-blocking vaccine efficacy. (a) Transmission-blocking effects of passively transferred immune sera on *P. yoelii* oocyst development in the *A. stephensi* mosquito midgut. Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before (Bf) or after (Af) intravenous injection of immune sera derived from mice i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone. Data are expressed as the median numbers of oocysts per mosquito (dots in boxes), quartiles (boxes), and ranges (lines above and below boxes). Statistically significant differences were found in all three immunization groups compared for the treatment after intravenous injection of immune sera ($P < 0.0001$) but not for the treatment before intravenous injection of immune sera ($P = 0.726$) in an analysis performed using the Kruskal-Wallis test. There are also statistically significant differences between the treatment before intravenous injection of immune sera and the treatment after intravenous injection of immune sera for all three immunization groups ($P < 0.0001$, Wilcoxon-Mann-Whitney U test). (b) Transmission-blocking effects of active immunization on oocyst development. Mice were i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone and then were infected with the parasite. Mosquitoes were allowed to directly feed on infected mice, the numbers of oocysts were determined, and the data were expressed as described above for panel a. Statistically significant differences were found in all three groups compared by using the Kruskal-Wallis test ($P < 0.0001$). Light microscopic images of representative mosquito midguts from the active vaccination experiments are shown at the bottom.

CT alone (median, 109 oocysts) or with Pys25 alone (median, 65 oocysts). Although we do not know why the CT or Pys25 immune serum had a significant parasite-killing effect (for CT, 346 oocysts versus 109 oocysts; for Pys25, 302 oocysts versus 65 oocysts), no mosquitoes completely lacked oocysts when they were given CT or Pys25 immune serum (Table 1). The results demonstrated that i.n. vaccination with Pys25 plus CT induced antibodies which confer complete transmission-blocking immunity when a passive vaccination regimen is used.

Next, to evaluate the direct mucosal vaccine efficacy of Pys25, mosquitoes were allowed to obtain a blood meal directly from parasite-infected mice that had been vaccinated as described in Materials and Methods. The results demonstrated that vaccination with Pys25 plus CT completely blocked oocyst development, as we observed in the passive vaccination experiment, while significant numbers of oocysts were observed in mosquitoes that fed on CT-vaccinated mice (median, 269 oo-

TABLE 1. Prevalence of oocyst infection in *A. stephensi* mosquitoes

Type of immunization	Vaccine	No. of oocyst-positive mosquitoes/total no. examined (% infection)[a]	No. of oocyst-positive mosquitoes/total no. examined (% infection)[a]	
			Before immune serum transfer[b]	After immune serum transfer[b]
Passive	CT		31/31 (100)	40/40 (100)
	Pys25		71/71 (100)	39/39 (100)
	Pys25 + CT		51/51 (100)	0/56 (0)
Active	CT	150/150 (100)		
	Pys25	90/90 (100)		
	Pys25 + CT	0/84 (0)		

[a] The percentages are the percentages of oocyst-positive mosquitoes based on the total numbers of mosquitoes examined.

[b] Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before or after intravenous transfer of immune sera obtained from mice i.n. vaccinated with Psy25 plus CT, with Psy25 alone, or with CT alone.

cysts) or Pys25-vaccinated mice (median, 170.5 oocysts) (Fig. 2b). Vaccination with Pys25 alone had a weak but significant transmission-blocking effect compared with the effect observed for CT-vaccinated mice, suggesting that i.n. vaccination with the recombinant antigen alone might have some efficacy, even though the antibody levels for these two groups were not significantly different (Fig. 1). The oocyst prevalence was 100% for all vaccination regimens that we tested except both the passive and active Pys25-plus-CT regimens, for which the oocyst prevalence was 0% (Table 1). On the basis of our results, we concluded that i.n. vaccination with the malaria OSP was very efficacious when this OSP was combined with a mucosal adjuvant to block parasite transmission to mosquitoes.

DISCUSSION

Although most vaccines in use today are administered s.c. or intramuscularly, the advantages of mucosal vaccines are indisputable; they result in local immunity as well as systemic immunity, which, in general, is hard for parenteral vaccines to induce, and they provide a first line of defense against many infections that occur at or emanate from mucosal surfaces. They could prevent transmission of blood-borne pathogens by reuse of syringes; they may be safer and more cost-effective and thus have advantages for developing countries; and they are painless and therefore likely to be readily tolerated by small children and individuals with needle phobia (16). Although not all of the advantages attributed to mucosal vaccines mentioned above are directly relevant to the design of vaccines against malaria, and although there are some intrinsic technical difficulties that cannot be circumvented by development of effective mucosal vaccines (7), evaluation of the concept of designing mucosal vaccines for nonmucosal pathogens seems to be worthwhile.

To investigate malaria parasite OSP-based mucosal vaccines, we previously demonstrated that for two types of human malaria (*P. falciparum* malaria and *P. vivax* malaria) experimentally induced mouse immune sera specific for Pfs25 and Pvs25 (homologues of rodent Pys25) were very effective in blocking parasite transmission from patients' parasitized blood to mosquitoes in a membrane feeding assay (2, 3). In the present study we demonstrated that in a rodent malaria infection model, OSPs were immunogenic when they were administered i.n. (with levels of antigen-specific Igs reaching 15 mg/ml), and the induced immune serum was very effective in blocking parasite transmission. Most importantly, however, we demonstrated that vaccination directly prevented the transmission of a parasite from vaccinated animals to feeding mosquitoes. To our knowledge, this is the first demonstration that mucosal vaccination with malaria OSPs can directly prevent malaria transmission to mosquitoes in vivo. In the DBA/2 strain of mice lacking a component of the complement system, the number of oocysts formed is significantly increased in feeding mosquitoes (26); however, anopheline mosquitoes collected in field are usually not as heavily infected, and a single oocyst is commonly detected. Therefore, a more moderate antibody level may confer effective transmission-blocking immunity in humans (21).

Malaria vaccines targeting hepatic and erythrocytic stages suffer from antigenic variations mainly due to selection pressure from the host immune system. However, antigens expressed at a parasite sexual stage, such as OSPs, are immunologically concealed from the host immune system, and hence the chance that antigenic variations occur may be low. Indeed, OSPs of *P. falciparum* and *P. vivax* were shown to have minimal antigenic variations even in field isolates collected from remote regions of the world (19, 27). This is an important characteristic of ideal vaccines. On the one hand, sexual-stage antigens have disadvantages such as (i) the absence of an infection-induced booster effect and the resulting long-term immunity and (ii) the absence of direct protection of vaccinees from infection. Therefore, it is believed that a vaccine candidate should have multiple components and that at least one component should be a sexual-stage antigen (7). In such a vaccine formulation, preerythrocytic and/or erythrocytic antigens may function cooperatively with sexual-stage antigens for prevention of or reduction of infection and parasite transmission.

Mucosal administration, such as i.n. or oral administration, unlike parenteral immunization, of nonreplicating inert antigens with CT tends to induce Th2-type immunity, which is characterized by predominant induction of serum IgG1, induction of local secretory IgA, and in some cases induction of serum IgE in mouse models. Unlike what happens in other infectious diseases, which require induction of cell-mediated immunity (22), serum antibody, regardless of the Ig isotype, seems to be the predominant, if not only, protective arm of immunity that blocks malaria transmission. We do not know the mechanism of action of Pys25-specific antibodies in blocking parasite development in the midgut of a feeding mosquito, but binding of antibodies to the zygote surface and subsequent prevention of parasite development into the ookinete may be the most important blocking mechanism (14, 25). This antibody binding may occur within the midgut, and this may be independent of Ig isotypes. Thus, although IgE antibody is not the major antibody isotype present in a vaccinated host, it may contribute to blocking transmission. However, the induction of serum IgE may potentially lead to an allergic response in vaccinated individuals, and the data shown in Fig. 1b are relevant to this issue. Another important issue that needs to be