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3D7-Derived *Plasmodium falciparum* Erythrocyte Membrane Protein 1 Is a Frequent Target of Naturally Acquired Antibodies Recognizing Protein Domains in a Particular Pattern Independent of Malaria Transmission Intensity

Louise Joergensen,* Lasse S. Vestergaard,* Louise Turner,* Pamela Magistrado,* John P. Lusingu,† Martha Lemnge,† Thor G. Theander,* and Anja T. R. Jensen2*

Protection against *Plasmodium falciparum* malaria is largely mediated by IgG against surface Ags such as the erythrocyte membrane protein 1 family (PIEMP1) responsible for antigenic variation and sequestration of infected erythrocytes. PIEMP1 molecules can be divided into groups A, B/A, B, C, and B/C. We have previously suggested that expression of groups A and B/A PIEMP1 is associated with severe disease and that Abs to these molecules are acquired earlier in life than Abs to PIEMP1 belonging to groups B, B/C, and C PIEMP1. In this study, we compared the acquisition of IgG to 20 rPIEMP1 domains derived from 3D7 in individuals living under markedly different malaria transmission intensity and were unable to find differences in the Ab acquisition rate to PIEMP1 of different groupings (A, B, or C) or domain type ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, or $\chi$). Abs were acquired early in life in individuals living in the high transmission village and by the age of 2–4 years most individuals had Abs against most constructs. This level of reactivity was found at the age of 10–20 years in the medium transmission village and was never reached by individuals living under low transmission. Nevertheless, the sequence by which individuals acquired Abs to particular constructs was largely the same in the three villages. This indicates that the pattern of PIEMP1 expression by parasites transmitted at the different sites was similar, suggesting that PIEMP1 expression is nonrandom and shaped by host-parasite relationship factors operating at all transmission intensities. *The Journal of Immunology*, 2007, 178: 428–435.

*individuals living in areas with high-intensity transmission of *Plasmodium falciparum* acquire protective immunity against malaria during childhood. This protection is probably largely mediated by IgG Abs specific for parasite-encoded variant surface Ags (VSA) expressed on the infected erythrocyte (IE) membrane. To date, the best characterized VSA family is the polymorphic *P. falciparum* erythrocyte membrane protein 1 (PIEMP1) (3). An important function of PIEMP1 is to mediate IE adhesion to specific host receptors on the vascular lining, enabling IE to sequester in various tissues and avoid splenic clearance. PIEMP1-mediated sequestration is considered a central element in the parasite’s attempt to evade host immunity and plays an important role in the pathogenesis of malaria (1, 4–8).

The PIEMP1 molecules of the *P. falciparum* clone 3D7 are encoded by 59 *var* genes (9), which can be grouped into three major groups (A, B, and C) and two intermediate groups (B/A and B/C) (9, 10). The extracellular and variable sequence of PIEMP1 comprises four different domain types: the N-terminal segment, the C2, the cysteine-rich interdomain region (CIDR), and the Duffy binding-like (DBL) domains (3, 9). The CIDR group has three ($\alpha$, $\beta$, $\gamma$) and DBL domains have seven ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, $\zeta$, $\chi$) distinct sequence classes (3, 11, 12). Groups A and B/A comprise the largest PIEMP1s, with a 7–10 domain structure, which is different from the 4-domain type structure predominant of groups B, B/C, and C (10).

VSA severe malaria (VSA$_{SM}$) expressed by parasites, causing severe *P. falciparum* malaria in young children with no or little protective immunity, appear serologically more conserved than VSA (VSA uncomplicated malaria (VSA$_{UM}$)) expressed by parasites causing uncomplicated and subclinical infection in older and more immune individuals (4, 6). Using Ab-selected and nonselected 3D7 sublines, we have previously established a link between expression of groups A and B/A PIEMP1 and a VSA$_{SM}$ phenotype and between expression of groups B, B/C, or C PIEMP1 and a VSA$_{UM}$ phenotype (13). Altogether, these findings imply that groups A and B/A PIEMP1 molecules are more frequently and better recognized by Abs acquired after natural infection than groups B, B/C, and C PIEMP1. In the present study, we have addressed this question and conclude that 3D7-derived PIEMP1 are targets of naturally acquired Abs which recognize different protein domains in a particular pattern, independent of malaria transmission intensity. However, we did not find any evidence for naturally acquired Abs being largely directed against particular groupings (A, B, or C) or domain types ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, or $\chi$) of PIEMP1.

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*Abbreviations used in this paper: VSA, variant surface Ag; IE, infected erythrocyte; PIEMP1, *P. falciparum* erythrocyte membrane protein 1; VSA$_{SM}$, VSA severe malaria; VSA$_{UM}$, VSA uncomplicated malaria; DBL, Duffy binding ligand; CIDR, cysteine-rich interdomain region.

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Table I. The 20 different 3D7-derived rPfEMP1 domains used in this study*.

<table>
<thead>
<tr>
<th>ups Type</th>
<th>Domain (var Gene)</th>
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<tbody>
<tr>
<td>upsA</td>
<td>DBL3β, DBL5β, CIDR1α (PF08_0107), DBL2β, DBL3γ, CIDR1γ, CIDR2β, C2 (PF13_0003), DBL1α, DBL3x, CIDR2β (PFD1235w)</td>
</tr>
<tr>
<td>upsB</td>
<td>DBL3β, DBL5β, DBL5x, C2 (MAL631.316), DBL1x (MAL775.55)</td>
</tr>
<tr>
<td>upsC</td>
<td>DBL2γ, CIDR1γ (PF08_0107), DBL1α (PFD0995c)</td>
</tr>
<tr>
<td>upsD</td>
<td>CIDR1α (PFB1055c)</td>
</tr>
</tbody>
</table>

* The domains represented all the different ups types in 3D7 (10), except upsE, which is found upstream of var2csa associated with pregnancy malaria (14).

Materials and Methods

Protein expression

A total of 20 different r3D7 protein domains representing PfEMP1 domains of all the different ups types in 3D7 (10), except upsE, found upstream of VAR2CSA associated with pregnancy-associated malaria (14), were cloned and expressed for ELISA. Slightly more upsA-type domains compared with other ups types were included in the study because previous studies had implied that Abs against these domains would be acquired early in life and that parasites expressing at least some of these domains would be virulent (13). Domains and subtypes of DBL (α, β, γ, δ, ε), C2, and CIDR (α, γ) were defined as described in Ref. 12 and specific primers for each recombinant domain were designed accordingly. The DBL1α domains (PFB1055c and PF13_0008), DBL3β and DBL5β (PFD1235w), CIDR1α (PFE1640w), and CIDR2β, and C2 (PF13_0003) were PCR amplified from 3D7 genomic DNA and subcloned into the pGEX-4T1 vector (GE Healthcare). The recombinant domains were expressed as fusion proteins at the C terminus of GST from Schistosoma japonicum (15) in Escherichia coli and purified by affinity chromatography on glutathione Sepharose 4B (GE Healthcare). DBL2β, DBL5x, C2 (MAL631.316), and DBL2x (PF08_0107) were subcloned into the pAcSecG2T baculovirus vector (BD Biosciences) for production of GST-tagged proteins. CIDR1α (PFD1235w and MAL775.55), DBL3x and CIDR2β (PFI1_0008), DBL2β and CIDR1γ (PF13_0003) were subcloned into the pBAD-TOPO vector (Invitrogen Life Technologies). The recombinant domains were expressed as fusion proteins at the C terminus of GST from Schistosoma japonicum (15) in Escherichia coli and purified by affinity chromatography on glutathione Sepharose 4B (GE Healthcare).

Results

Ab recognition of PfEMP1 depends on malaria transmission intensity and age

It has previously been shown that acquisition of Abs to VSA depends on transmission intensity and shows an age-related buildup (6–8, 18). Using 20 different 3D7-derived rPfEMP1 domains representing all the different ups types in 3D7 (10), except the upsE VAR2CSA associated with pregnancy malaria (14) (Table I), we compared IgG levels in plasma from individuals aged 2–19 years, living under high (Mgome), moderate (Ubiri), and low (Magamba) malaria transmission intensity (Fig. 1). In children 2–4 and 5–9 years, and young adolescents 10–14 years of age, median Ab levels to PfEMP1 were significantly higher in Mgome than in corresponding age groups living in Ubiri and Magamba villages (p < 0.05; Dunn’s all pairwise multiple comparison). Similarly, median Ab levels were significantly higher in individuals living in Mgome village aged 15–19 years as compared with individuals of the same age group living in Magamba (p < 0.05). The 10- to 14-year-old children and the 15–19 year adolescents from Ubiri had significantly higher median plasma Ab levels to r3D7 PfEMP1 domains in individuals living in three villages (A) Mgome, (B) Ubiri, and (C) Magamba of high, moderate, and low malaria transmission intensity, respectively. For each age group (2–4, 5–9, 10–14, and 15–19 years), including 15 individuals, Ab levels were calculated as described in Materials and Methods. The median Ab level was categorized into four different intervals: 0–10 (white), 11–20 (light gray), 21–60 (dark gray), and >61 (black).
Ab levels compared with the two age-matched groups living in Magamba ($p < 0.05$).

Within Mgome and Magamba villages, we were unable to find any statistically significant difference in the median Ab level between the four different age groups (Fig. 1, A and C). In Ubiri, we found a significantly higher median Ab level in plasma from individuals aged 15–19 years as compared with plasma from children 2–4 and 5–9 years of age ($p < 0.05$; FIGURE 2. Percentage of individuals from a high malaria transmission area (Mgome) having plasma Abs recognizing recombinant expressed 3D7 PfEMP1 of different ups type. Each age group (2–4, 5–9, 10–14, and 15–19 years) included 15 individuals. A, DBL3b, DBL5a, and CIDR1a of PFD1235w (upsA). B, DBL2b, DBL3y, CIDR1y, CIDR2b, and C2 of PF13_0003 (upsA). C, DBL1a, DBL3x, CIDR2b of PF11_0008 (upsA). D, DBL2b, DBL5e, and C2 of MAL6P1.316 (upsBsh). E, CIDR1a of MAL7P1.55 (upsBsh). F, DBL1a of PFB1055c (upsB). G, DBL2b and CIDR1γ of PF08_0107 (upsC). H, DBL1α of PFD0995c (upsC). I, CIDR1α of PFE1640w (upsD). ups nomenclature according to Ref. 10.
Dunn's all pairwise multiple comparison). Similarly, young adolescents (10–14 years) living in Ubiri had a significantly higher median Ab level (p < 0.05) as compared with children 5–9 years (Fig. 1B).

Specific ups or domain types do not predict Ab recognition

The 1.5-kb nontranslated 5’ region of 3D7 var genes has previously been classified into upsA-E, upsBsh, upsBsh+, upsB+, and...
upsC" (9, 10). In accordance with this classification, we grouped our 20 recombinant protein domains into upsA, upsB, upsBsh, upsB", upsC", upsD and looked for differences in the percentage of individuals recognizing protein domains encoded by var genes with a specific ups 5' region such as upsA, but were unable to find any (Figs. 2–4). In addition to the grouping of the 5' region of the different var genes, the CIDR can be grouped as three (α, β, γ) and DBL domains as seven (α, β, γ, δ, ε, ζ, ξ)
distinct sequence classes (12). We tested whether there was any significant difference in the Ab recognition of the different sequence classes, but again were unable to find any (Figs. 2–4).

The Ab-recognition pattern of different PfEMP1 domains predicts the recognition pattern in succeeding age groups in low-high malaria transmission villages

The Ab recognition by plasma from individuals of different ages living in a high (Mgome), moderate (Ubiri), and low (Magamba) transmission area was correlated for each individual village using the Spearman rank order correlation. For each age group in each village, the 20 domains were ranked from 1 to 20 according to how high a percentage of individuals had Abs to the domain. Each point in the graphs compare the ranking of a particular domain in two age groups. The Ab-recognition pattern was compared in: A–C1, children aged 2–4 years and children aged 5–9 years; A–C2, children aged 2–4 years and children aged 10–14 years; A–C3, children aged 2–4 years and adolescents aged 15–19 years; A–C4, children aged 5–9 years and children aged 10–14 years; A–C5, children aged 5–9 years and adolescents aged 15–19 years; A–C6, young adolescents 10–14 years and adolescents aged 15–19 years. Rs, Spearman rank order correlation coefficient.

Table II. Naturally acquired Abs recognize 20 3D7 PfEMP1 domains in a pattern independent of malaria transmission intensity

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</tr>
</thead>
<tbody>
<tr>
<td>Ubiri (2–4)</td>
<td>0.516b</td>
<td>0.380</td>
<td>0.511</td>
<td>0.523</td>
<td>0.787</td>
<td>0.619</td>
<td>0.661</td>
<td>0.770</td>
</tr>
<tr>
<td>Ubiri (5–9)</td>
<td>0.0199</td>
<td>0.0960</td>
<td>0.0211</td>
<td>0.0179</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Ubiri (10–14)</td>
<td>0.0510</td>
<td>0.0371</td>
<td>0.0216</td>
<td>0.00774</td>
<td>0.00109</td>
<td>0.000</td>
<td>0.000</td>
<td>0.00428</td>
</tr>
<tr>
<td>Ubiri (15–19)</td>
<td>0.610</td>
<td>0.664</td>
<td>0.591</td>
<td>0.752</td>
<td>0.544</td>
<td>0.638</td>
<td>0.632</td>
<td>0.504</td>
</tr>
<tr>
<td>Mgome (2–4)</td>
<td>0.575</td>
<td>0.632</td>
<td>0.475</td>
<td>0.423</td>
<td>0.00207</td>
<td>0.00122</td>
<td>0.000404</td>
<td>0.0708</td>
</tr>
<tr>
<td>Mgome (5–9)</td>
<td>0.6081</td>
<td>0.00281</td>
<td>0.0339</td>
<td>0.0623</td>
<td>0.512</td>
<td>0.731</td>
<td>0.532</td>
<td>0.431</td>
</tr>
<tr>
<td>Mgome (10–14)</td>
<td>0.00211</td>
<td>0.00000</td>
<td>0.0157</td>
<td>0.0564</td>
<td>0.666</td>
<td>0.763</td>
<td>0.564</td>
<td>0.498</td>
</tr>
<tr>
<td>Mgome (15–19)</td>
<td>0.00338</td>
<td>0.00000</td>
<td>0.00319</td>
<td>0.0128</td>
<td>0.623</td>
<td>0.806</td>
<td>0.626</td>
<td>0.546</td>
</tr>
</tbody>
</table>

PIEMP1 constructs were ranked from 1 to 20 according to the percentage of individuals having Abs to the domains; the ranks were analyzed using the Spearman rank test.

Spearman rank order correlation coefficient (rs).

Value of p.
pattern by plasma from individuals aged 15–19 years (Fig. 5B3), the recognition pattern of any particular age group was found to predict the recognition pattern of plasma from all succeeding age groups within the same village (Fig. 5).

Naturally acquired Abs recognize specific PfEMP1 domains in a pattern independent of malaria transmission intensity

Of the eight different PfEMP1 molecules, five were represented by two or more domains (see Materials and Methods). The percentage of individuals having plasma Abs recognizing, e.g., DBLβ of PFD1235w did not predict recognition (Spearman rank order correlation) of any of the two other PFD1235w domains tested. Similarly, none of the cloned MAL6P1.316, PF08_0107, PF11_0008, and PF13_0003 domains were able to predict recognition of domains from within the same PfEMP1 (Figs. 2–4 and data not shown).

To analyze whether PfEMP1 domains are recognized in a specific pattern independent of transmission intensities, we correlated the recognition pattern obtained in the three different villages (Figs. 2–4). PfEMP1 constructs were ranked from 1 to 20 according to the percentage of donors having Abs to the domains. Ranks obtained were analyzed and the 20 different 3D7 domains were found to be recognized in a similar pattern in all three villages, as indicated by the Spearman rank order coefficients and p values given in Table II.

Discussion

P. falciparum infections can be controlled by Abs acquired following natural exposure to the parasite (19). The main targets of these Abs are believed to be VSA expressed on the surface of IE (8, 20, 21). Previous publications suggest VSA-specific immune responses to impose a restriction on the repertoire of variant Ags compatible with parasite survival and to drive expression from VSA_{SM} toward VSA_{UM}, during the early years of childhood (4, 6, 22). VSA_{SM} appears to be serologically less diverse than VSA_{UM} (7), consistent with the observation that immunity to severe malaria is acquired more rapidly than to uncomplicated disease and subclinical infection (23). Using Ab-selected and nonselected 3D7 sublines, we have previously established a link between expression of groups A and B/A PfEMP1 and a VSA_{SM} phenotype and between expression of groups B, C, or C PfEMP1 and a VSA_{UM} phenotype. In particular, we have proposed a relationship between a VSA_{SM} phenotype and expression of a 3D7 group A var encoded PfEMP1 PFD1235w/MAL8P1.207 (13). This proposed relationship implies the product of this gene to be recognized at high levels and early in life of individuals living in malaria endemic areas. We have previously published data supporting this relationship (13) and in addition recently shown the presence of IgG Abs to one domain (CIDR1α) of PFD1235w/MAL8P1.207 to be associated with a reduced risk of febrile malaria attacks and anemia during natural infections in malaria endemic areas (24).

In this study, we tested the hypothesis that groups A (upsA 5′ region) and B/A PfEMP1 (upsBsh ′ 5′ region) are more frequently recognized by plasma from individuals living in malaria endemic areas than groups B (upsB 5′ region), B/C (upsBsh 5′ region), and C (upsC and upsC′ 5′ region) molecules.

The intensity, seasonality, and stability of transmission of P. falciparum play an important role in the development of immunity to malaria (25), as well as in the clinical epidemiology of the disease (26, 27). In this study, we used samples from three villages of different malaria transmission intensity. In the high malaria transmission village (Mgome), infants and small children carried most of the malaria burden with a high P. falciparum prevalence and parasite density compared with villagers in the sites with moderate (Ubiri) and low (Magamba) transmission (16). Correspondingly, Abs to the 20 different r3D7 PfEMP1 domains were acquired at much faster rates in Mgome than in Ubiri and Magamba indicating that acquisition of Abs depends on the intensity of malaria transmission. This correlation between malaria transmission intensity and acquisition of Abs is in line with our recent finding of Abs to PFD1235w/MAL8P1.207 in a cohort of 320 individuals from two different Tanzanian villages of high (Mkokola) and moderate (Kwamasimba) malaria transmission (24). In high malaria transmission areas, immunity to malaria develops earlier in life compared with areas of less transmission. Thus, a broad VSA-specific IgG repertoire with high levels of Abs comparable to that of adults would be expected even in young children in areas of high malaria transmission intensity. By contrast, in a low transmission area a narrow VSA-specific repertoire with low levels of Abs would be expected in young children as well as adults, because infection only happens very rarely and the immune system does not get stimulated nor boosted. As supported by our data from Mgome and Magamba, this would in both cases become reflected in no or slight differences in Ab levels among different age groups. By contrast, in a moderate transmission area with development of immunity and induction of Abs to different VSA occurring more slowly than in a high malaria transmission area, but yet faster than in a low transmission area with a stochastic infection risk, a measurable progression in the development of Abs is to be expected as indicated by the age-dependent increase in Abs to PfEMP1 domains seen in individuals living in Ubiri.

As in our previous publications, most protein domains of the PFD1235w/MAL8P1.207 gene were well-recognized by young children as well as adolescents (13, 24), but we did not find any support for our hypothesis of group A (upsA 5′ region) or B/A (upsBsh ′ 5′ region) being more frequently recognized by individuals as compared with group B (upsB 5′ region), C (upsC and upsC′ 5′ region) or B/C (upsBsh 5′ region) PfEMP1 variants. Likewise, there was no evidence that any distinct sequence class (CIDRα, β, or γ and DBLα, β, γ, δ, ε, or x) was better recognized than others. None of the plasma donors are likely to have been infected with 3D7 never the less the 20 different 3D7 protein domains were recognized by plasma Abs from most individuals naturally exposed to other P. falciparum clones, clearly indicating that there is serological cross-reactivity between PfEMP1 expressed by different parasites. The recognition of one particular protein domain encoded by a specific var gene (e.g., MAL6P1.316, PFD1235w/MAL8P1.207, and PF13_0003) did not predict the percentage of Ab responders to other domains encoded by the same var gene. Taken together, this indicates the presence of PfEMP1 variants with similarity to 3D7-encoded domains in field isolates of P. falciparum and indicates exchange of single to few domain blocks within var genes rather than preservation of full-length PfEMP1 within parasite populations. It has previously been suggested that such exchange is more likely to take place between groups B and C PfEMP1, but that some characteristics of group A PfEMP1 have leaked into these two groups (10). Based on the present and previous studies (24, 29), we speculate certain PfEMP1 domains (e.g., CIDR) to be more likely exchanged and conserved than others and thus to contribute to the VSA_{SM} phenotype of parasites causing severe malaria in individuals with little or no immunity. Such conservation and exchange of certain domains would explain the discrepancy between the results presented here and those previously seen in comparison between severe and uncomplicated malaria (13), because for a significant finding to occur, severe malaria-type domains and not full-length PfEMP1 would need to be identified before testing of a much larger number of different domain types and would probably also require a larger...
set of plasma samples from different individuals. The current grouping of var genes is largely based on the analysis of the 3D7 genome (10) and it is likely that a more extensive analysis including full genome sequences currently becoming available from The Sanger Institute and Broad Institute will identify subgroups within the established groupings (28). Thus, it is conceivable that our statistical analyses comparing domains based on the current grouping is too simple in biological terms. Thus, our inability to detect differences between Ab recognition of domain groups in this study does not rule out the possibility that severe malaria-type PFEIP1 domains can be predicted on the basis of var gene analysis and grouping.

In the high as well as moderate and low malaria transmission villages, we found the Ab-recognition pattern of the 20 different PFEIP1 domains by any particular age group (age span 2–19 years) to predict the recognition pattern by plasma from all succeeding age groups. In addition, this Ab-recognition pattern was found to be similar between the three Tanzanian villages. This indicates that PFEIP1 variants expressed by parasites transmitted under different transmission intensities contain homologous domains and that Abs to these domains are acquired at fixed sequence. Thus, the order by which Abs were acquired was the same in the three villages, even though they were acquired at a faster rate with increased transmission. This finding indicates that the repertoire of PFEIP1 molecules or other PFEIP1 domains expressed by parasites at the three sites was similar and suggests PFEIP1 expression to be nonrandom and shaped by factors governing the host-parasite relationship that operates at all levels of transmission.

Acknowledgments
We are grateful to all study participants, parents/guardians, as well as village helpers and health management teams in Tanzania. We thank Lotte Bram and Susanne Pedersen for excellent technical assistance.

Disclosures
The authors have no financial conflict of interest.

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