Expression of *Plasmodium falciparum* 3D7 STEVOR proteins for evaluation of antibody responses following malaria infections in naïve infants

N. SCHREIBER[1], A. KHATTAB[2], M. PETTER[2], F. MARKS[1,4], S. ADJEI[3], R. KOBBE[1], J. MAY[1] *and* M.-Q. KLINKERT[2]*

[1] *Infectious Disease Epidemiology Group, Bernhard-Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany*
[2] *Department of Molecular Medicine, Bernhard-Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany*
[3] *Kumasi Centre for Collaborative Research in Tropical Medicine, KNUST University Post Office, Kumasi, Ghana*
[4] *International Vaccine Institute, Kwanak PO Box 14, Seoul 151-600, South Korea*

(*Received 19 June 2007; revised 30 July and 19 August 2007; accepted 20 August 2007; first published online 12 October 2007*)

SUMMARY

Clinical immunity to *Plasmodium falciparum* malaria develops after repeated exposure to the parasite. At least 2 *P. falciparum* variant antigens encoded by multicopy gene families (*var* and *rif*) are targets of this adaptive antibody-mediated immunity. A third multigene family of variant antigens comprises the *stevor* genes. Here, 4 different *stevor* sequences were selected for cloning and expression in *Escherichia coli* and His_6–tagged fusion proteins were used for assessing the development of immunity. In a cross-sectional analysis of clinically immune adults living in a malaria endemic area in Ghana, high levels of anti-STEVOR IgG antibody titres were determined in ELISA. A cross-sectional study of 90 nine-month-old Ghanaian infants using 1 recombinant STEVOR showed that the antibody responses correlated positively with the number of parasitaemia episodes. In a longitudinal investigation of 17 immunologically naïve 9-month-old infants, 3 different patterns of anti-STEVOR antibody responses could be distinguished (high, transient and low). Children with high anti-STEVOR-antibody levels exhibited an elevated risk for developing parasitaemia episodes. Overall, a protective effect could not be attributed to antibodies against the STEVOR proteins chosen for the study presented here.

Key words: recombinant STEVOR, *Plasmodium falciparum*, immune responses, infants.

INTRODUCTION

Plasmodium falciparum malaria is one of the most devastating diseases in sub-Saharan Africa. Amongst the individuals living in malaria endemic areas, children under the age of 5 years are at highest risk of morbidity and mortality. The development of anti-*P. falciparum* immune responses in young children is still poorly understood. Clinical immunity to malaria in older children and adults, rarely sterile, develops after continuous or successive exposure to parasites. While there is evidence to suggest that existing antibodies can protect from infection with distinct parasite strains, it is believed that only responses against a few antigens mediate protection from disease (Bull *et al*. 1998).

Variant surface antigens (VSA) are known to be the major targets of naturally acquired antibodies (Roberts *et al*. 1992; Bull *et al*. 1998; Ofori *et al*. 2002). Most extensively studied are PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1) antigens, present in knobs on the infected erythrocyte surface and encoded by the *var* multicopy gene family (Su *et al*. 1995). Two other families in the same subtelomeric regions close to the *var* genes are the *rif* (repetitive interspersed family) and the *stevor* (subtelomeric variable open reading frame) gene families. RIFIN proteins were first identified via surface iodination and immunoprecipitation by immune sera (Helmby *et al*. 1993; Fernandez *et al*. 1999). These clonally variant antigens have also been reported as targets of the human immune system (Abdel-Latif *et al*. 2002, 2003; Schreiber *et al*. 2006).

STEVOR and RIFIN proteins appear to be evolutionarily related, sharing sequence and structural similarities, possibly also related functionalities (Cheng *et al*. 1998; Dzikowski *et al*. 2006; Lavazec *et al*. 2006). Immunofluorescence and immunoelectron microscopic studies have localized STEVOR to Maurer's clefts (MC), which are flattened membranous structures, believed to be partly responsible for transporting parasite proteins to the erythrocyte surface (Kaviratne *et al*. 2002). Like

* Corresponding author: Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany. Tel: +49 40 42818301. Fax: +49 40 42818400. E-mail: mo.klinkert@bni-hamburg.de

 © 2007 Cambridge University Press
doi:10.1017/S0031182007003794 Printed in the United Kingdom

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

source: https://doi.org/10.48350/26970 | downloaded: 19.4.2024

PfEMP1, STEVORs may also be trafficked to the surface (Lavazec *et al*. 2006). The observation that synthetic STEVOR peptides can bind to human erythrocytes is suggestive of an involvement in rosette formation (Garcia *et al*. 2005). Based on the predicted protein structure with a highly variable loop between two transmembrane regions and its expression patterns in parasite blood stages, these proteins are speculated to be involved in antigenic variation as a means of immune evasion (Lavazec *et al*. 2006, 2007). Expression of STEVORs in different stages in the *Plasmodium* life-cycle (Florens *et al*. 2002), trophozoites (Kaviratne *et al*. 2002) and gametocytes (Sutherland 2001; McRobert *et al*. 2004; Sharp *et al*. 2006) indicates their potential importance for parasite survival (McRobert *et al*. 2004).

We have carried out a first analysis of representative members of the STEVOR protein family towards understanding their biological significance, with an emphasis on immunological aspects. So far nothing is known about immune responses against STEVOR proteins in malaria patients. Here, we evaluated *P. falciparum* STEVOR proteins by cloning and expressing them in *Escherichia coli* and investigated the induction of anti-STEVOR antibodies to the recombinant proteins after malarial infections, in order to further our understanding of the immunological roles of members of this multicopy gene family in the parasite. Antibody responses to different recombinant STEVOR proteins were first examined in a cross-sectional study of clinically immune African adults. Correlation between the frequency of *P. falciparum* infections and the level of anti-STEVOR IgG in 9-month-old infants was sought. Further, the development of anti-STEVOR antibodies following first acute parasitaemia episodes in immunologically naïve children was assessed longitudinally.

MATERIALS AND METHODS

PCR amplification and cloning

DNA was extracted from the *Plasmodium falciparum* reference strain 3D7 with the QIAamp MiniKit (QIAGEN, Hilden, Germany). The 4 *stevor* genes used in the study were selected based on evidence of transcription, with a focus on those that were widely expressed over several life-cycle stages. Thus, the PFA0750w gene was shown to be transcribed in almost all parasite stages including the asexual, sexual and liver stages in oligo-microarray studies of the reference strain 3D7 (Le Roch *et al*. 2003). In the same study, slightly narrower transcription patterns were observed for the *stevor* genes PFL2610w and PFC0025c. The MAL13P1.7 gene was shown to be transcribed in the late ring to early trophozoite stages of the 3D7 and HB3 parasites in 2 other oligo-microarray studies (Bozdech *et al*. 2003; Llinas *et al*. 2006). In these studies, the first 3 *stevor* genes were also transcribed in both ring and trophozoite stages.

Some N- and C-terminal nucleotide sequences representing signal peptide and predicted transmembrane region, respectively, were excluded from PCR amplification, to avoid their possible interference during bacterial expression and in subsequent purification procedures. The following primer sets were used: PFA0750w forward (5′ – GTA AGT TTC ATT CAA AAC AAC ACC A – 3′) and PFA0750w reverse (5′ – GCC CAT AGC AGC AAA AGC AGC A – 3′); PFL2610w forward (5′ – GAG AAT TAT CTA AAT AAC CAT TAT A – 3′) and PFL2610w reverse (5′ – CGA AGT TGC AGC AGG TAC TGT TAA- 3′); MAL13P1.7 forward (5′ – GTA CAA AAC AAC ACC AAA AGA ACA A – 3′) and MAL13P1.7 reverse (5′ – CAT AGC AGC ACC TGC AGC AGT T – 3′); PFC0025c forward (5′ – GTA AGT TTC ATT CAA AAC AAC ACC A – 3′) and PFC0025c reverse (5′ – CGA AGC AGA AGT TGC ACC TGA T – 3′). PCR products were purified and thereafter ligated into the pTrcHis2-TOPO® vector (Invitrogen, Karlsruhe, Germany). Positive clones were identified by colony screening-PCR and sequences determined using the AGOWA sequencing service (Berlin, Germany) with Invitrogen vector primers pTrcHis forward (5′ – GAG GTA TAT ATT AAT GTA TCG – 3′) and pTrcHis reverse (5′ – GAT TTA ATC TGT ATC AGG – 3′).

Protein expression, purification and production of polyclonal antibodies

The recombinant plasmids were transformed in TOP10 *Escherichia coli* bacteria and *stevor* sequences expressed as His_6-STEVOR fusion proteins according to the pTrcHIS2-TOPO® TA Expression Kit protocol (Invitrogen). Recombinant proteins were purified to 90% homogeneity, according to the protocol described for the preparation of proteins expressed in inclusion bodies under denaturing conditions (QIAexpressionist™ handbook, QIAGEN). Proteins were analysed in 12·5% SDS-polyacrylamide gels and their identity verified in immunoblots using commercial anti-His6-antibodies (QIAGEN). Protein concentration was determined by standard Bradford protein assay (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Purified proteins (50 μg of each) were used for immunizing NMRI mice to obtain polyclonal anti-STEVOR antibodies.

Parasite cell culture, antigen preparation and immunoblotting

P. falciparum parasites were isolated from the peripheral blood of 3 children from our study area, diagnosed with severe malaria infections. The field

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

isolates were adapted to laboratory conditions, and maintained under continuous culture according to standard protocols (Ljungström *et al*. 2004). Crude *P. falciparum* lysate was prepared as previously described (Schreiber *et al*. 2006).

Parasite material used for immunoblotting was prepared from trophozoites of the 3D7 *P. falciparum* strain as well as a number of laboratory-adapted field isolates, the origin of which was Gabon for Chc03 (Lindenthal *et al*. 2003) and Gb337 (Khattab *et al*. 2004) and Senegal for VIP43 (Khattab *et al*. 2004). MACS-enriched parasite-infected erythrocytes were diluted at a concentration of 1×10^6 infected erythrocytes per μl in 10 mM HEPES, pH 7·2, containing protease inhibitor mix M (Serva, Heidelberg) and subjected to hypotonic lysis by repeated freezing and thawing in liquid nitrogen. The lysate was centrifuged for 10 min at 14 000 ***g*** to separate the membrane fraction from the soluble proteins, and the pellet containing an enriched membrane fraction was washed twice with PBS. Proteins were extracted in 2× SDS sample buffer at 95 °C for 5 min for separation by SDS-PAGE. Proteins were transferred to Trans-Blot transfer medium membrane (Bio-Rad, Hercules, CA) and probed with polyclonal mouse anti-STEVOR-antibodies (1 : 2000) followed by goat-anti-mouse-HRP antibodies (1 : 30 000, Pierce Biotechnology, Rockford, IL). Blots were developed with enhanced chemiluminescence (ECL; Amersham Biosciences).

Phylogenetic analysis

STEVOR-deduced amino acid sequences of the *P. falciparum* 3D7 reference strain were obtained from PlasmoDB (www.plasmodb.org). A total of 35 sequences were used for multiple sequence alignment construction by the ClustalW software integrated into The Biology WorkBench web-based tool for biologists (http://workbench.sdsc.edu/). The alignment was used as an input for ClustalTREE to generate an unrooted phylogenetic tree using the Neighbour-Joining method. The tree was bootstrapped 100 times for deriving confidence values for the groupings in the tree. A bootstrap value over 70 was considered reliable. The constructed tree was drawn using the MEGA 3 tree explorer. Identities between pairs of STEVOR-deduced amino acid sequences were calculated from the distance matrix generated by ClustalW using the formula '1-distance'.

Enzyme-linked immunosorbent assay (ELISA)

IgG reactivity to the respective antigen was determined via ELISA. Optimal antibody and antigen concentrations were determined in checkerboard titrations for both sets of adult and children plasma samples. ELISAs were repeated in 2 independent experiments and all sera were tested together for each antigen at one time, in order to control for day-to-day and test-to-test variations. Each plasma sample was tested in duplicate and in a third well that contained no antigen but plasma only, serving as blank. Samples were re-tested if the standard deviation (S.D.) of the duplicate OD values was $>0{\cdot}05$. In addition, to account for inter-assay variations, in each plate a positive and a negative pool of plasma was tested, comprising samples from 13 malaria-exposed Ghanaian adults and 10 non-exposed European adults, respectively. A cut-off value for anti-STEVOR-IgG levels was determined from the values obtained for individual European plasma samples, calculated as mean relative units (RU) ± 3 S.D. Test samples with an RU value above the calculated cut-off were classified as positive.

Microtitre plates with 96 wells (NUNC maxisorb, Wiesbaden, Germany) were coated with 50 μl of each of the STEVOR antigens or crude *P. falciparum* lysate at a concentration of 0·5 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9·5). Plates were incubated overnight at 4 °C and washed 4 times with washing buffer (0·5% Tween 20, phosphate buffered saline (PBS)) using the TECAN Genesis Workstation 200 pipetting robotics (TECAN, Crailsheim, Germany). Thereafter they were blocked with 200 μl of blocking buffer (4% bovine serum albumin (BSA), PBS) per well for 5 h at room temperature (RT). Plasma samples from adults and children were diluted 1 : 250 and 1 : 300, respectively, in washing buffer containing 1% BSA. Diluted antibody solutions (50 μl) were added to each well and incubated at 4 °C overnight. Plates were then washed as described above. Peroxidase-conjugated goat antibody (F(ab′)2 fragment) recognizing the γ-chains of human IgG (50 μl, diluted 1 : 15 000; Jackson Immunoresearch, Soham, Cambs, UK) was added to each well and further incubated (1 h RT). After repeated washing, 50 μl per well of tetramethyl benzidine peroxidase substrate (BD Biosciences, Franklin Lakes, USA) were added. The reaction was stopped after 5 min (for adults) and 10 min (for infants) with 50 μl of 0·5 M H_2SO_4. Absorbance was measured at 450 nm (GENios, TECAN).

RU was calculated as follows:

$$\frac{\textit{mean } OD_{450}(\textit{test sample}) - \textit{blank }(\textit{test sample})}{\textit{mean } OD_{450}(\textit{positive pool}) - \textit{blank }(\textit{positive pool})} \times 100 = RU$$

Study area and populations

Two studies (a cross-sectional and a longitudinal) were performed in the Ashanti Region in Ghana, which is holoendemic for malaria. The area has 2 rainy seasons: a major one extending from April to

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

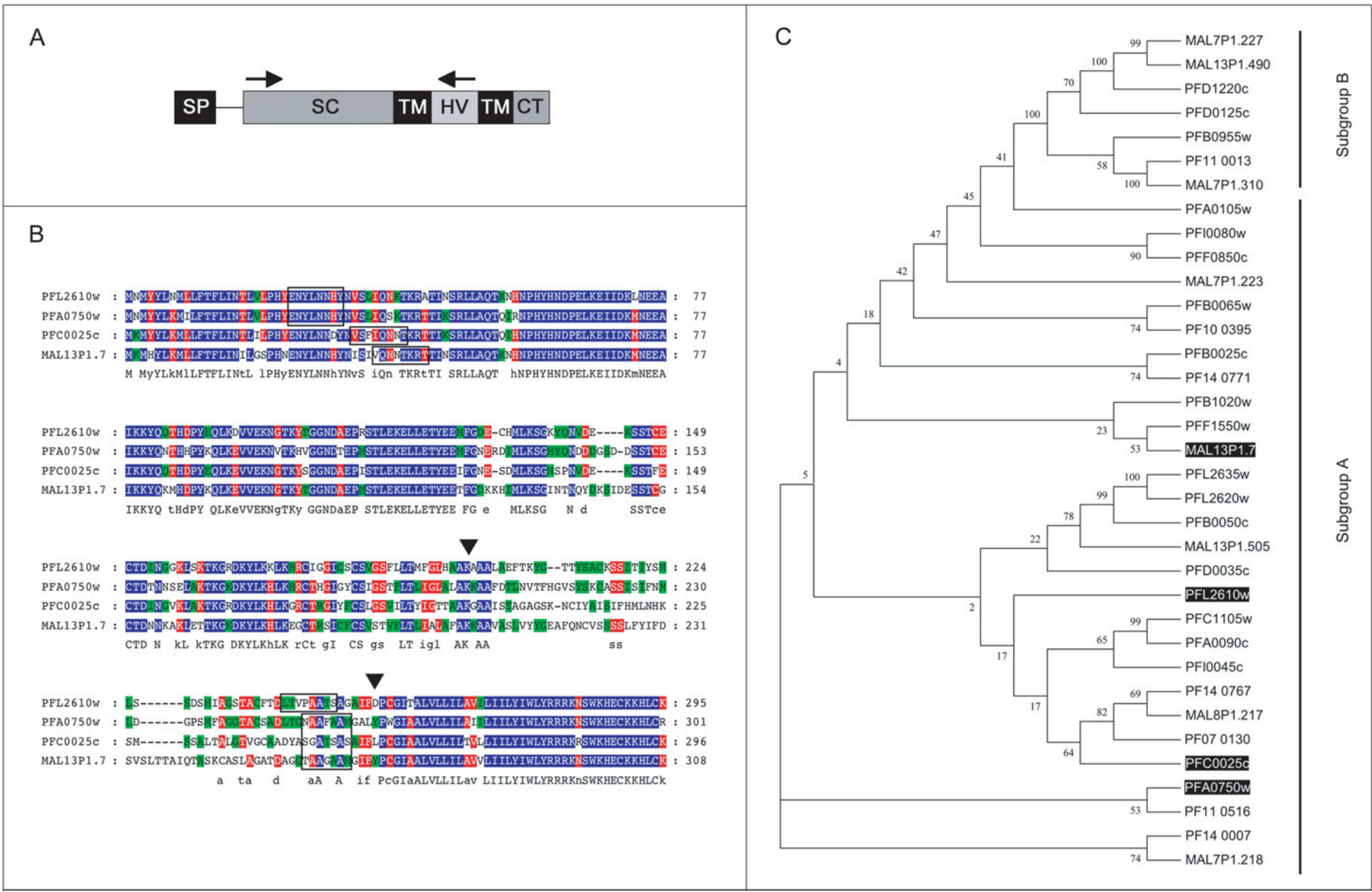

Fig. 1. For legend see opposite page.

August and a minor one from October to November. The study participants were considered fairly homogenous in being subjected to the same seasonal variation of infection prevalence (22·7% in the rainy and 13·5% in the dry season) (Kobbe *et al*. 2006).

A first investigation was based on the cross-sectional study and aimed at comparing IgG immune responses against 4 recombinant STEVOR proteins. To this end, plasma samples were collected from 195 adults, who lived in a small rural village during the year 2003. The majority of the participants were farmers, traders and teachers, amongst them were 83 couples sharing the same households.

Two further investigations were based on samples collected longitudinally during the course of a clinical trial (Kobbe *et al*. 2007). Briefly, 1070 infants were enrolled from 9 villages in the region at the age of 3 months and, given the involvement of a large number of participants, recruitment lasted from January to December 2003. Episodes of *P. falciparum* parasitaemia and clinical malaria were monitored monthly by active follow-up visits, in addition to passive case detection. All episodes of clinical malaria were treated with artesunate (4 mg/kg/dose)/amodiaquine (10 mg/kg/dose) for 3 days, the former having a short half-life of 43 min (Newton *et al*. 2000), while the active metabolite of the latter decays more slowly with a half-life of over 100 h (Winstanley *et al*. 1987). Malaria episodes detected within 21 days after anti-malarial drug treatment were not considered as new infections (Kobbe *et al*. 2007). Blood samples were taken every 3 months, beginning at the age of 3 months until the children reached the age of 24 months. Children were not included in the study if they showed clinical signs of malnutrition or HIV infections at the time of enrolment.

For the second investigation, study participants were selected from infants in the placebo group who were neither HbS nor HbC carriers ($n=385$). The individuals were first grouped according to whether they had malaria, parasitaemia or no parasitaemia. For the purpose of this investigation, we further subgrouped them according to their number of parasitaemia episodes as follows (i) 30 children who had no observed parasitaemia or malaria when they were 3 to 9 months old; (ii) 22 children with one observed episode of parasitaemia between the ages of 3 to 9 months of age; (iii) 38 children with more than 1 observed episode of parasitaemia between the ages of 3 and 9 months. The plasma samples from these infants taken at the age of 9 months were used.

For the third investigation, which aimed at analysing the development of anti-STEVOR-IgG responses in the first 2 years of life, infants were subjected to a number of additional selection criteria. First, all plasma samples for IgG determination collected at 3-monthly intervals during the monitoring period from 9 to 24 months had to be available (a total of 6). Second, data of at least 14 of the 16 monthly active follow-up visits had to be available. Third, the infants had to be free of any observed *P. falciparum* infections from the time they were first monitored at 3 months until they reached the age of 9 months. Of the 65 infants who met the above criteria, we further excluded those who were hospitalized during their third to ninth month of life, as well as those who had detectable anti-*P. falciparum*-IgG antibodies at the age of 9 months (i.e. those who were not immunologically naïve to *P. falciparum*). Only 17 participants met the criteria for our analysis.

For all studies described here, ethical clearance was obtained through the Ethics Committee of the School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Written informed consent was obtained from each participant or their parent or guardian.

Statistical analyses

Analyses were performed with Stata v9.2 (StataCorp, College Station, Texas). Differences between continuous non-normally distributed variables were assessed by two-sided Mann-Whitney and Kruskal-Wallis tests and values with $P<0·05$ considered significant. Correlation was assessed using Spearman's rank correlation coefficient.

RESULTS

Analysis of stevor sequences

P. falciparum DNA from 3D7 parasites was amplified by PCR using pairs of custom designed primers for PFA0750w, PFL2610w, MAL13P1.7 and PF-C0025c *stevor* genes. The overall STEVOR protein organization is structurally very similar to that of RIFINs (Fig. 1A) (Blythe *et al*. 2004; Dzikowski

Fig. 1. (A) STEVOR protein organization. STEVOR proteins comprise a signal peptide (SP) encoded by the first exon, followed by a semi-conserved (SC) region and a hyper-variable (HV) region. The latter is flanked by 2 predicted transmembrane (TM) domains (Cheng *et al*. 1998). Following the second TM is a short C-terminal region (CT). Expressed regions are delineated by the arrows. The map is not drawn to scale. (B) Protein sequence alignment of STEVOR variants. Sequence homologies are highlighted in blue (for 100% identity), in red (75% identity) and in green (50% identity). Amino acids on which primers were constructed are framed. The HV region is marked with arrowheads. (C) Phylogenetic tree of 3D7 STEVOR proteins. Two major subgroups termed A and B could be classified. STEVOR variants used in this study are highlighted as black boxes. Bootstrap values are indicated in the tree.

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

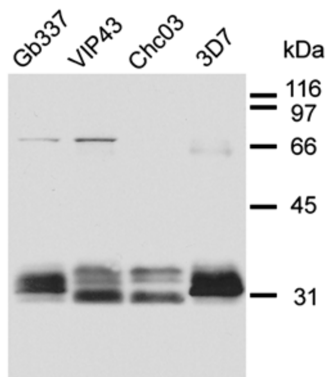

Fig. 2. Western blot analysis. Polyclonal mouse anti-STEVOR-antibodies raised against the 4 different variants (shown for MAL13P1.7) recognize different native STEVOR proteins in the enriched membrane fractions of different laboratory-adapted field isolates (Gb337, VIP43, Chc03) as well as the *Plasmodium falciparum* reference strain 3D7.

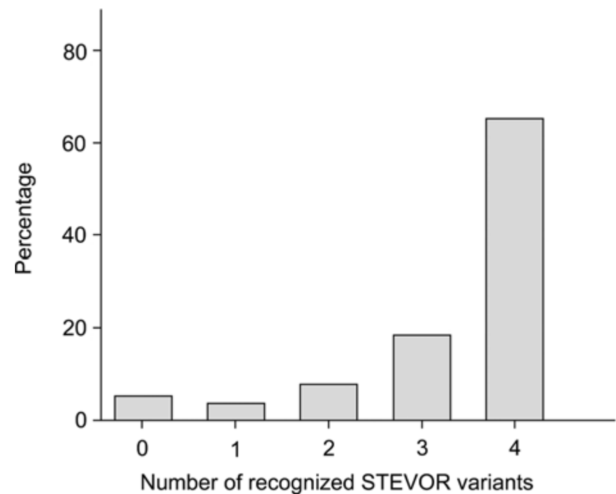

Fig. 3. Frequency of recognition of recombinant proteins by human sera. The percentage of sera containing antibodies to 0, 1, 2, 3 or 4 STEVOR variants was determined. Positive test samples are those with an RU above the cut-off value, calculated as the mean of RU ± 3 S.D. of the European controls.

et al. 2006). Multiple amino acid sequence alignment of the 4 *stevor* genes using the ClustalW software was performed (Fig. 1B). A comparison of the entire cloned sequences indicates high amino acid sequence conservation between 68 and 75%, which is the consequence of identical amino acids present in the long semi-conserved (SC) region and the shorter C-terminal region (CT) that follows the second transmemebrane (TM) region. Considerably lower amino acid sequence identities (33–47%) span the hypervariable (HV) domains, delineated by arrowheads shown in Fig. 1B.

An analysis of the database showed that 34 of the 35 3D7 full-length deduced STEVOR sequences are between 241 and 308 amino acids long, the exception being the $\gamma\beta$ STEVOR isoform encoded by the PFB0050c gene, which has only 172 amino acids. In contrast to the SC domain, the HV domain is short and comprises only 35–72 residues. Phylogenetic analysis of all 35 deduced amino acid sequences enabled us to classify the STEVOR proteins into 2 tentative subgroups (Fig. 1C). The larger one, which we refer to as subgroup A, is composed of 28 sequences. Due to the low bootstrap values, further subdivision into defined clusters was not possible. The STEVOR sequences selected for this study are highlighted in black boxes and, in being not too closely clustered, we considered them as representatives of divergent variants.

The remaining 7 protein sequences make up the smaller subgroup B and are characterized by STEVOR-like features. Here, the first 4 sequences in the tree determine STEVORs which show a lower than usual degree of conservation in the SC part of the protein compared to other STEVORs. In addition, together with 5 other sequences belonging to subgroup A, the encoded proteins are predicted to carry only 1 TM domain, unlike the other STEVORs, which are predicted to have 2. The other 3 so-called degenerate *stevor* genes encode proteins that are distinguished by even greater sequence degeneracy in their SC domains.

Both groups of *stevor* genes are distributed over various chromosomes. According to the PlasmoDB nomenclature, PFA0750w is located on chromosome 1, PFC0025c on chromosome 3, PFL2610w on chromosome 12 and MAL13P1.7 on chromosome 13. Both subgroups also contain variants whose genes have orientations towards both the centromere and the telomere. The 4 variants which we have chosen for our analysis are all orientated towards the telomere.

Recognition of native P. falciparum *proteins by anti-STEVOR antisera*

Based on previous demonstrations of a membrane association of STEVOR proteins (Przyborski *et al*. 2005), antisera produced in mice against the 4 His_6-STEVOR fusion proteins were analysed in an immunoblot of an enriched erythrocyte/parasite membrane fraction prepared by mechanical lysis in hypotonic solution of different *P. falciparum* strains. Antibodies were found to react with distinct bands in different parasite strains, sizes of which are compatible with the calculated molecular masses of STEVOR proteins (between 23·8 and 36·5 kDa) (Fig. 2). All 4 antisera exhibited the same recognition pattern, shown exemplarily for antiserum raised against the MAL13P1.7 recombinant product. In the enriched membrane fraction prepared from Gb337 parasites, the antisera reacted with 2 protein bands 32 and 34 kDa in size. In both VIP43 and Chc03 parasites, antisera detected a 30 kDa band, in

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

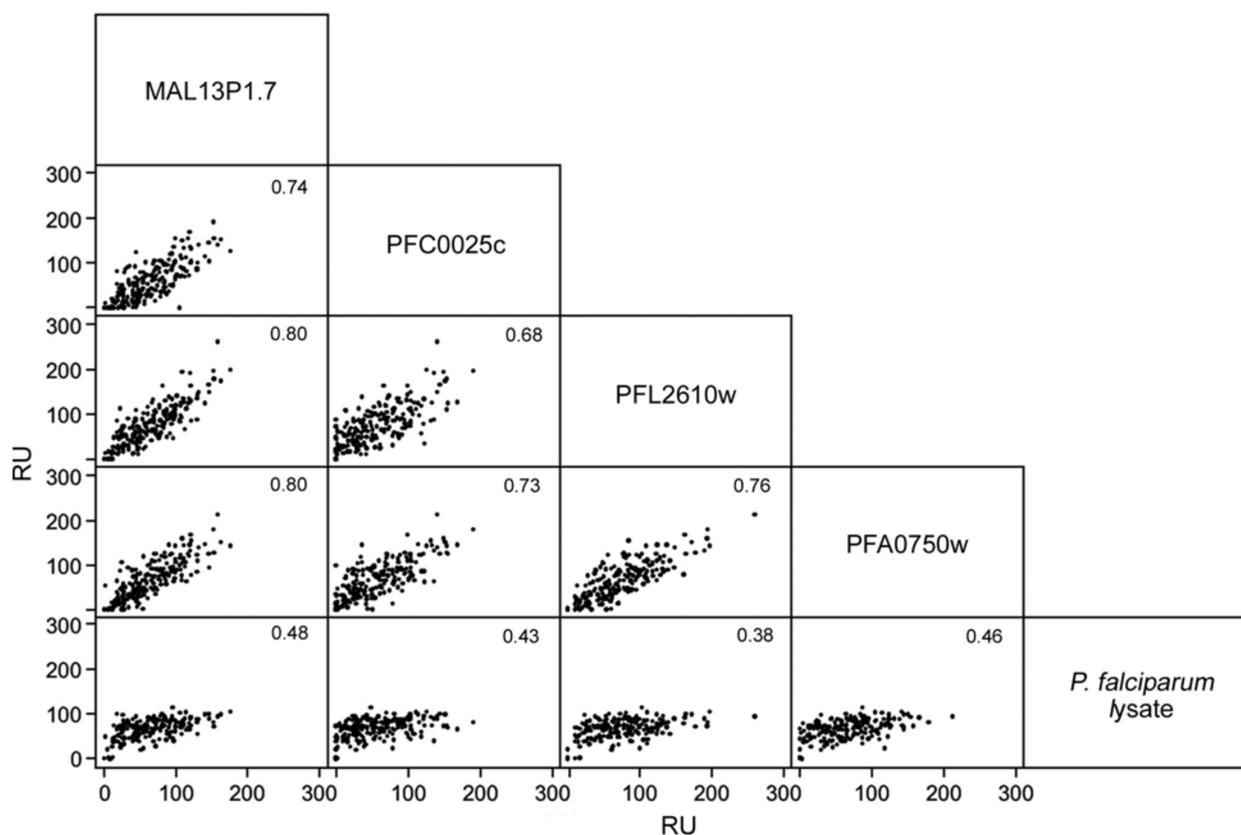

Fig. 4. Dot plots of anti-STEVOR-IgG responses. IgG levels against different recombinant STEVOR variants and against a *Plasmodium falciparum* lysate were determined in 195 African adults by ELISA and shown in a pairwise comparison. Spearman's rank correlation coefficient values (*rho*) are indicated in each box.

addition to 2 weaker reacting bands, approximately 33 and 35 kDa in size. In 3D7 parasites, a doublet very similar in size to that in Gb337 parasites was observed. Pre-immune sera were tested in parallel and found to be negative (data not shown).

The finding that all antisera gave a similar recognition pattern may be explained by the high degree of conservation in a large part of the recombinant proteins, and in sharing many common epitopes, thus eliciting antibodies with similar specificities. Since the variants studied here are capable of generating antibodies that recognize an array of native STEVOR proteins expressed in different parasite strains, we conclude that our recombinant STEVOR proteins are relevant for detecting anti-STEVOR antibodies in human sera via ELISA and that all 4 are equally suited for this analysis.

Immune responses to different STEVOR variants in clinically immune African adults

To investigate anti-STEVOR antibody responses in malaria-exposed individuals, the recombinant STEVOR proteins were tested in ELISA using plasma samples collected from our cross-sectional study. The frequency of recognition of the recombinant proteins was determined and 65% of the adult sera were observed to recognize all 4 fusion proteins, whereas only 5% of these sera did not react with any of them (Fig. 3). Extending our immunological analysis, correlation between recognition of different STEVORs was sought. Results of pairwise recognition are depicted in dot plots, showing overall good correlation (*rho* values in the order of 0·68 to 0·80) (Fig. 4). Most sera reacted with each of the STEVOR proteins to a similar extent, but there were also some that reacted differently with the 4 proteins.

In parallel to the anti-STEVOR antibody determinations, immune responses to a crude *P. falciparum* lysate were analysed, serving as a reference marker for previous malaria infections. Since antigens differ in their capacity to stimulate the immune system and to induce antibody production, the observed weak correlation between antibodies against a single STEVOR and *P. falciparum* lysate, containing a huge repertoire antigens, was not unexpected (*rho* values ranging from 0·38 to 0·48) (Fig. 4).

Antibody responses in infants with different numbers of preceding infections

Samples from the investigation encompassing 90 children of 9 months were used to analyse the naturally occurring immune responses in infants. In these small children, the total IgG levels against *P. falciparum* lysate increased significantly with an

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

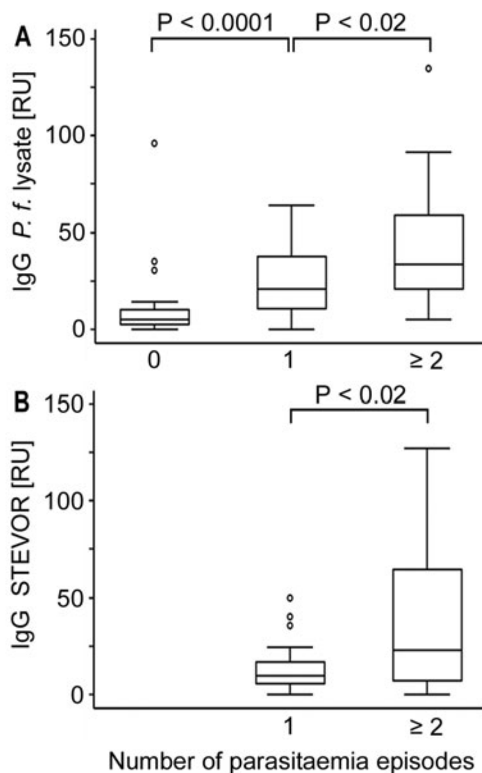

Fig. 5. Comparison of median (interquartile range) antibody levels with parasitaemia episodes. IgG levels against total *Plasmodium falciparum* antigens and recombinant STEVOR protein encoded by the PFC0025c gene were determined by ELISA. The participants were divided according to their accumulated number of parasitaemia episodes during their 3rd to 9th months of life. The 3 groups were those with no detectable parasitaemia episodes ($n=30$), one ($n=22$) and two or more ($n=38$). *P* values were calculated using the Mann-Whitney test.

increasing number of preceding infections (Fig. 5A). Anti-*P. falciparum* antibodies were detected in 3 of the 30 individuals in the group with no observed *P. falciparum* infections during the follow-up period. Antibodies to *P. falciparum* extracted antigens have been found in non-exposed adults and also occur in children and infants with no or minimal exposure to malaria (Belizario *et al*. 1997). However, to investigate the likelihood of undetected infections in these individuals, we sought to analyse previous plasma samples taken at months 3 and 6. In child 1, the RU values for the anti-*P. falciparum*-lysate responses at months 3, 6 and 9 were 58·9, 24·5 and 30·2, respectively. In child 2, RU values increased from 18·4 (at month 3) to 29·9 (at month 6) and 95·8 (at month 9), and in child 3 RU values were measured at 22·4, 11·7 and 34·9. Most probably, maternal IgG levels were present at the age of 3 months, but expected to have been eliminated by the age of 6–9 months, based on knowledge that the half-life time of IgG antibodies is between 7 and 21 days. We therefore argue that persistent or increasing antibody levels measured at month 9 in these infants are the consequence of previously unobserved asymptomatic *P. falciparum* infections.

In view of the negative immunoreactivity measured in the majority of children in the subgroup with no previous episodes of parasitaemia (Fig. 5A) analysis of anti-STEVOR responses was only performed in infants with preceding episodes of parasitaemia. Due to limited availability of serum samples, only 1 *stevor* gene product encoded by PFC0025c was used. This product was chosen mainly because of its availability at the time of study. Similar results as with the *P. falciparum* lysate were observed, in that the anti-STEVOR IgG levels also increased significantly with the number of infections (Fig. 5B).

Antibody profiles in a longitudinal follow-up study

One principle question was whether anti-STEVOR antibodies detected with our reagents are associated with a symptomatic or an asymptomatic course of infection. We therefore analysed anti-STEVOR response profiles of infants and correlated the anti-STEVOR responses to parasitaemia episodes. The longitudinal investigation distinguished 3 different patterns based on the relationship between antibody responses and infection episodes (Fig. 6). Four individuals (numbers 44, 871, 875, 935) were assigned to a distinct pattern with an increase in anti-STEVOR IgG levels after first infection and high levels of antibodies were measured until the end of follow-up (classified here as 'type I'). Frequent reinfections or recrudescences could explain the multiple bouts of parasitaemia (6–10 episodes). A different pattern was seen in 5 children (numbered 66, 123, 447, 516 and 868) characterized by a peak of anti-STEVOR antibody responses, which disappeared to basal levels after the transient increase (classified as 'type II'). These children had fewer parasitaemia events than 'type I' individuals. STEVOR responses in 8 children were characterized by low or undetectable anti-STEVOR IgG levels which remained at almost undetectable levels through to 24 months, even after patients had been parasitaemic (classified here as 'type III' profile which can be further divided into IIIa and IIIb types, see below).

In children with the type I profile, a similar pattern was observed for antibody responses against *P. falciparum* lysate and STEVOR. In individuals with a transient STEVOR response (type II), anti-*P. falciparum* antibodies appeared after preceding parasitaemia. In STEVOR low responders (type III), 2 subgroups with different anti-*P. falciparum* antibody profiles could be distinguished. Individuals with type IIIa profile (labelled 226, 450, 650, 757) showed low anti-STEVOR and anti-*P. falciparum* responses after parasitaemia. In contrast, children with type IIIb profile (labelled 631, 733, 787, 844)

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

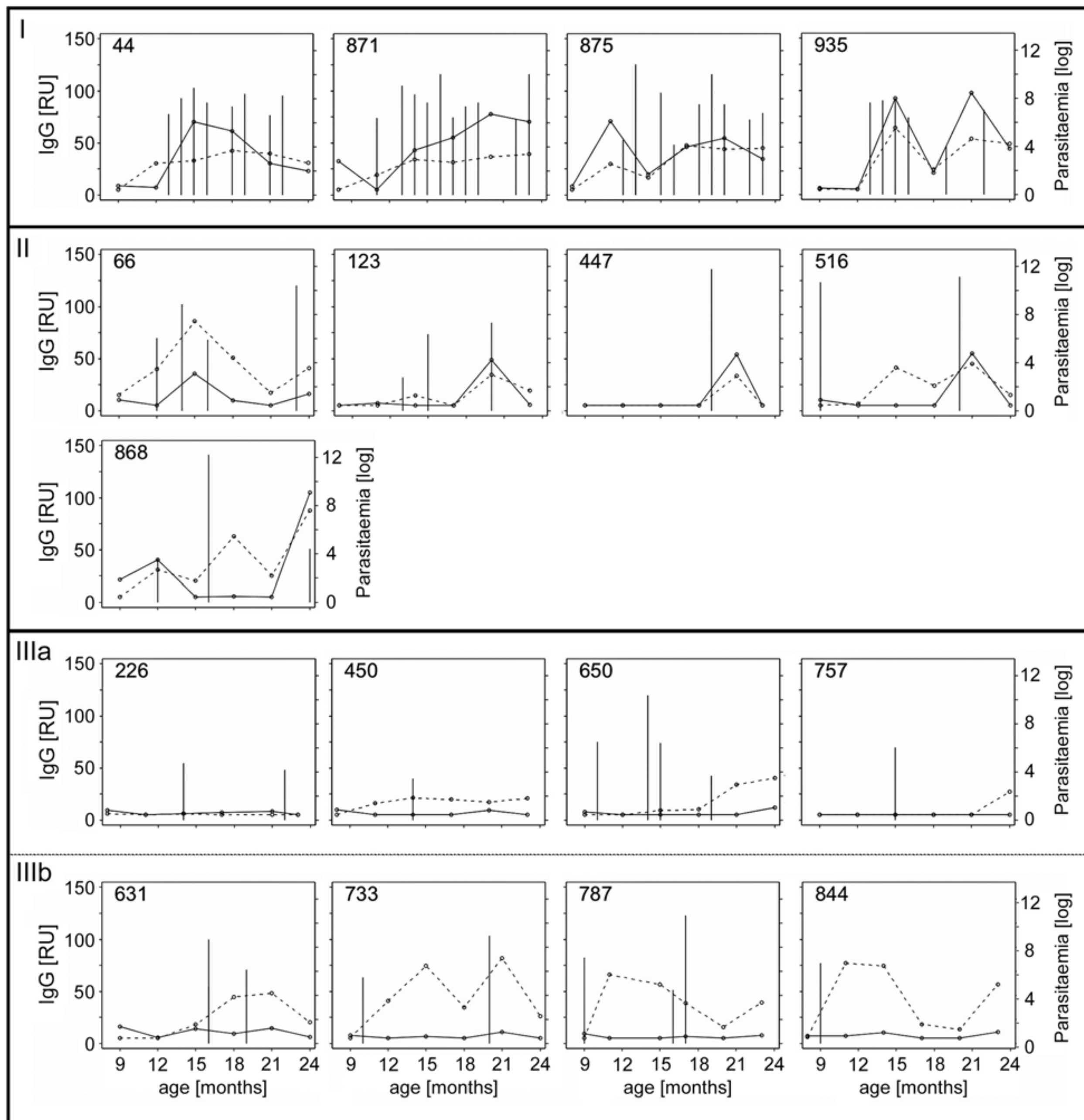

Fig. 6. Antibody profiles observed during follow-up. Plasma samples were taken every 3 months and analysed for anti-STEVOR- (solid line) and anti-*Plasmodium falciparum*- (dotted line) antibodies by ELISA, expressed as RU (relative units) based on measured OD values. Vertical solid lines illustrate the time-points when the patients become parasitaemic, the height of the line representing the parasite density (calculated as the logarithm of parasitaemia).

remained non-responsive to the STEVOR variant, but showed recognition of parasite lysate proteins.

Correlation of parasitaemia and anti-STEVOR IgG levels

We next analysed the effect of parasitaemia episodes on IgG levels during the following month and, vice versa, the effect of IgG levels on episodes of parasitaemia in the following month. Data collected from the third investigation were used for this analysis and the scheme is shown in Fig. 7. Children with parasitaemia episodes had significantly higher anti-STEVOR antibody titres determined in the following month than children without parasitaemia ($P<0{\cdot}001$). There was no difference whether the parasitaemic children were symptomatic or asymptomatic ($P<0{\cdot}87$) (Table 1). On the other hand, the level of anti-STEVOR IgG did not protect individuals from becoming parasitaemic in the following month. Instead, children with elevated anti-STEVOR IgG levels even had a significantly higher probability of parasitaemia episodes in the following month ($P<0{\cdot}001$, Table 1). In contrast, elevated anti-*P. falciparum* IgG levels were not associated with a higher risk for the development of a

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

Table 1. Statistical analysis of parasite and IgG determinations

(Anti-STEVOR IgG responses were measured by ELISA in 17 children every 3 months from 9 to 24 months of age. A total of 85 events were theoretically available for analysis (5 visits in 17 children). This number (*n*) was accordingly reduced if follow-up visits were missing, so that 84 events were available for the first analysis (effect of parasitaemia on IgG at months 12, 15, 18, 21 and 24) and 81 for the second analysis (effect of IgG at months 9, 12, 15, 18 and 21 on parasitaemia). There was a period of 1 month between parasite determination and IgG determination or vice versa. *P* values were calculated using the Mann-Whitney test.)

		n	Anti-STEVOR-IgG median [RU]	25–75 percentile	*P* value <
Effect of parasitaemia on IgG	No parasitaemia	65	4·7	1·5–9·0	0·001
	Parasitaemia	19	42·9	10·6–55·2	
	– asymptomatic	10	44·4	19·8–54·6	0·87
	– symptomatic	9	35·8	10·6–55·2	
Effect of IgG on parasitaemia	No parasitaemia	55	5·2	1·5–9·5	0·002
	Parasitaemia	26	12·2	7·1–45·9	
	– asymptomatic	16	11·5	7·4–40·8	0·87
	– symptomatic	10	20·5	4·6–61·2	

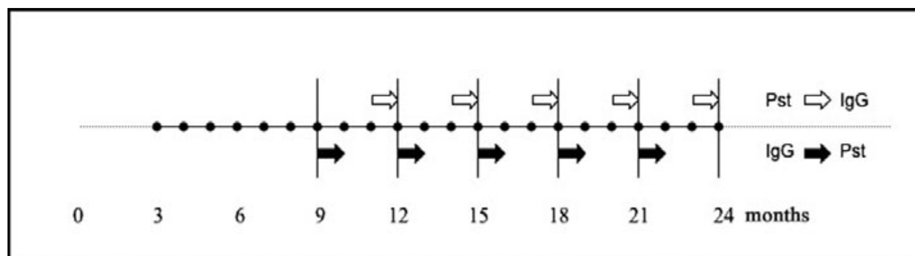

Fig. 7. Scheme of the longitudinal study. Children were monitored on a monthly basis (black dots) starting at the age of 3 months through to 24 months. Blood samples were taken at 3-monthly intervals (indicated by vertical lines) for IgG determinations against STEVOR and *Plasmodium falciparum* lysate. The scheme further distinguishes (i) the assessment of the occurrence and outcome of parasitaemia (Pst), detected 1 month prior to IgG determination on anti-STEVOR antibody levels (open horizontal arrow above the time-line) from (ii) the assessment of anti-STEVOR antibody levels on Pst (solid horizontal arrow below the time-line).

parasitaemia episode in the following month (data not shown). It is improbable that antibodies against STEVOR mediate higher susceptibility to plasmodial infections. Elevated anti-STEVOR-IgG levels in children with higher rates of parasitaemia episodes are in all likelihood due to a generally higher risk of these children to become parasitaemic.

DISCUSSION

One important aspect in the evaluation of parasite antigens as potential components of a subunit malaria vaccine is the understanding of naturally acquired immunity. Asexual blood-stage vaccine candidates include those encoded by single copy genes, such as merozoite surface proteins (MSP) 1–3, the apical membrane antigen 1 (AMA-1), the ring-stage infected erythrocyte surface antigen (RESA), the glutamate-rich protein (GLURP), serine repeat antigen (SERA) and the erythrocyte-binding antigen (EBA-175) (Girard *et al*. 2007). A vaccine candidate has also been developed based on a highly variable protein from the multicopy gene family encoding PfEMP-1 molecules (Ahuja *et al*. 2006). In this study we have analysed the antibody-mediated response to proteins of the third largest multigene family of variant antigens, encoded by *stevor* genes.

In a first analysis, elevated antibody levels against different recombinant STEVOR variants were observed in malaria-exposed adults. Although dot plots indicated strong pairwise correlations between the sera in their recognition of recombinant antigens, there were also some adult sera capable of discriminating between the proteins. Originating from adults living in an endemic area, the sera most probably contain multiple antibody specificities for the different variant types. However, it is also reasonable to assume that most of the elicited antibodies would be dictated by the SC domain, given that this region spans a large portion of the protein. Because of its postulated topology (the SC domain being intracellular) it is expected that antibodies to these

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

internal epitopes are elicited only after rupture of parasites and are not likely to be relevant in protection. The observation in our immunoblots that antisera raised against the recombinant proteins recognize protein bands of identical mobility in different parasite strains supports the above assumption that antibodies in human plasma samples might also recognize epitopes in the SC domain.

Keeping in mind that the variation of STEVOR proteins is a likely immune evasion mechanism, future studies could be directed at an analysis of the HV domains to determine a role in malaria immunity. For example, work would entail expression in recombinant forms of HV domains of STEVOR variants of a given parasite, and the use of a larger longitudinal study cohort to follow antibody persistence to single variants. The limitation of this approach is the hypervariability between paralogues and across isolates, despite the fact that a few inter-parasite variants still share high homology over this region (data not shown).

To investigate the primary immune response to the STEVOR protein family, we chose 9-month-old infants who were immunologically naïve to plasmodial infections and in whom most probably maternally-derived antibodies no longer play a role in their protection from malaria infection. Despite limitations in our strategy to use a single recombinant protein and despite the small number used for the analysis, being the first of its kind, some novel observations have nevertheless emerged. The longitudinal study demonstrated that the presence of anti-STEVOR IgG antibodies does not protect individuals from becoming parasitaemic. In some children, the rapid increase in and the perpetuation of the anti-STEVOR titres appeared to be associated with the frequency (6–10) of parasitaemia episodes during the observation period (type I profile), leading us to conclude that anti-STEVOR antibodies serve as a marker of infection in our experimental system rather than a marker of protection. Transient increase of anti-STEVOR responses was mostly seen directly after parasitaemia episodes, with STEVOR responses appearing to be short-lived (type II profile). There are probably several explanations for the lack of antibody responses in the type III profiles. It can be speculated that there is insufficient competition of STEVOR antigens with other more immunogenic proteins for presentation to the immune system. Additionally, variant-specific recognition of STEVOR antigens is also possible, for example some of these individuals may not be infected with parasite clones expressing a STEVOR with sufficient antigenic similarity to PFC0025c. Human genetic factors may also determine why an individual is a non-responder, even though most of the literature known to date shows that responses to variant antigens are not genetically determined (Bull *et al*. 2005). Nonetheless, sickle cell trait associated with enhanced IgG antibody responses to variant surface antigens has been recently reported (Cabrera *et al*. 2005). In our investigations HbC and HbS carriers were excluded.

In the context of our small longitudinal study, conclusions drawn on any possible role of anti-STEVOR responses measured in this study in malaria protection are conceivably premature. A general function in promoting disease or decreasing protection is unlikely, and the role of anti-STEVOR antibodies in malaria remains to be investigated. Mathematical models suggesting the existence of major and minor epitopes on VSAs propose that long-lasting immune responses against major epitopes of a specific variant are capable of preventing the re-emergence of this variant in a following infection. In contrast, short-lived and partially cross-reactive immune responses against minor epitopes of VSAs might promote chronic (possibly asymptomatic) infections (Recker *et al*. 2004). While antibodies against these minor epitopes are not capable of removing the parasite expressing the variant in question, they could conceivably limit and thus prolong its growth, thereby sustaining a chronic infection. Field studies indicate that in older children the duration of infection increases while the density of parasitaemia and the number of malaria attacks decline (Molineaux and Gramiccia, 1980). It has also been shown that children with a higher multiplicity of infection are more likely to have asymptomatic infections (Beck *et al*. 1997). Together with the observations of our study that anti-STEVOR responses are short-lived and that elevated antibody levels are associated with repeated episodes of parasitaemia, it is tempting to propose that STEVORs, as part of the machinery of antigenic variation, are constituents of minor epitopes and contribute mainly in eliciting transient immune responses.

We are very grateful to all study participants, their parents and guardians. We also thank Yu-Shan Chia for invaluable advice on protein expression, Robin Kobbe for guidance with the study cohorts and Jürgen Sievertsen for technical advice. The work is part of the Ph.D. thesis of N.S. The study was funded by grants from the Bundesministerium für Bildung und Forschung (01KA0202), Friends of the Bernhard Nocht Institute for Tropical Medicine and the Chica and Heinz Schaller Foundation. S.A. was supported by the German Academic Exchange Service.

REFERENCES

Abdel-Latif, M. S., Dietz, K., Issifou, S., Kremsner, P. G. and Klinkert, M.-Q. (2003). Antibodies to *Plasmodium falciparum* rifin proteins are associated with rapid parasite clearance and asymptomatic infections. *Infection and Immunity* **71**, 6229–6233.

Abdel-Latif, M. S., Khattab, A., Lindenthal, C., Kremsner, P. G. and Klinkert, M.-Q. (2002). Recognition of variant Rifin antigens by human antibodies induced during natural *Plasmodium*

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

falciparum infections. *Infection and Immunity* **70**, 7013–7021.

Ahuja, S., Pettersson, F., Moll, K., Jonsson, C., Wahlgren, M. and Chen, Q. (2006). Induction of cross-reactive immune responses to NTS-DBL-1alpha/x of PfEMP1 and *in vivo* protection on challenge with *Plasmodium falciparum*. *Vaccine* **24**, 6140–6154.

Beck, H. P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P. and Tanner, M. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66. *The Journal of Infectious Diseases* **175**, 921–926.

Belizario, V. Y., Saul, A., Bustos, M. D., Lansang, M. A., Pasay, C. J., Gatton, M. and Salazar, N. P. (1997). Field epidemiological studies on malaria in a low endemic area in the Philippines. *Acta Tropica* **63**, 241–256.

Blythe, J. E., Surentheran, T. and Preiser, P. R. (2004). STEVOR–a multifunctional protein? *Molecular and Biochemical Parasitology* **134**, 11–15.

Bozdech, Z., Zhu, J., Joachimiak, M. P., Cohen, F. E., Pulliam, B. and DeRisi, J. L. (2003). Expression profiling of the schizont and trophozoite stages of *Plasmodium falciparum* with a long-oligonucleotide microarray. *Genome Biology* **4**, R9.

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I. and Marsh, K. (1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nature Medicine* **4**, 358–360.

Bull, P. C., Pain, A., Ndungu, F. M., Kinyanjui, S. M., Roberts, D. J., Newbold, C. I. and Marsh, K. (2005). *Plasmodium falciparum* antigenic variation: relationships between *in vivo* selection, acquired antibody response, and disease severity. *The Journal of Infectious Diseases* **192**, 1119–1126.

Cabrera, G., Cot, M., Migot-Nabias, F., Kremsner, P. G., Deloron, P. and Luty, A. J. (2005). The sickle cell trait is associated with enhanced immunoglobulin G antibody responses to *Plasmodium falciparum* variant surface antigens. *The Journal of Infectious Diseases* **191**, 1631–1638.

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M. and Saul, A. (1998). *Stevor* and *rif* are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Molecular and Biochemical Parasitology* **97**, 161–176.

Dzikowski, R., Templeton, T. J. and Deitsch, K. (2006). Variant antigen gene expression in malaria. *Cellular Microbiology* **8**, 1371–1381.

Fernandez, V., Hommel, M., Chen, Q., Hagblom, P. and Wahlgren, M. (1999). Small, clonally variant antigens expressed on the surface of the *Plasmodium falciparum*-infected erythrocyte are encoded by the *rif* gene family and are the target of human immune responses. *The Journal of Experimental Medicine* **190**, 1393–1404.

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B., Tabb, D. L., Witney, A. A., Wolters, D., Wu, Y., Gardner, M. J., Holder, A. A., Sinden, R. E., Yates, J. R. and Carucci, D. J. (2002). A proteomic view of the *Plasmodium falciparum* life cycle. *Nature, London* **419**, 520–526.

Garcia, J. E., Puentes, A., Curtidor, H., Vera, R., Rodriguez, L., Valbuena, J., Lopez, R., Ocampo, M., Cortes, J., Vanegas, M., Rosas, J., Reyes, C. and Patarroyo, M. E. (2005). Peptides from the *Plasmodium falciparum* STEVOR putative protein bind with high affinity to normal human red blood cells. *Peptides* **26**, 1133–1143.

Girard, M. P., Reed, Z. H., Friede, M. and Kieny, M. P. (2007). A review of human vaccine research and development: malaria. *Vaccine* **25**, 1567–1580.

Helmby, H., Cavelier, L., Pettersson, U. and Wahlgren, M. (1993). Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. *Infection and Immunity* **61**, 284–288.

Kaviratne, M., Khan, S. M., Jarra, W. and Preiser, P. R. (2002). Small variant STEVOR antigen is uniquely located within Maurer's clefts in *Plasmodium falciparum*-infected red blood cells. *Eukaryotic Cell* **1**, 926–935.

Khattab, A., Reinhardt, C., Staalsoe, T., Fievet, N., Kremsner, P. G., Deloron, P., Hviid, L. and Klinkert, M.-Q. (2004). Analysis of IgG with specificity for variant surface antigens expressed by placental *Plasmodium falciparum* isolates. *Malaria Journal* **3**, 21.

Kobbe, R., Kreuzberg, C., Adjei, S., Thompson, B., Langefeld, I., Thompson, P. A., Abruquah, H. H., Kreuels, B., Ayim, M., Busch, W., Marks, F., Amoah, K., Opoku, E., Meyer, C. G., Adjei, O. and May, J. (2007). A randomised controlled trial of extended intermittent preventive antimalarial treatment in infants. *Clinical Infectious Diseases* **45**, 16–25.

Kobbe, R., Neuhoff, R., Marks, F., Adjei, S., Langefeld, I., von Reden, C., Adjei, O., Meyer, C. G. and May, J. (2006). Seasonal variation and high multiplicity of first *Plasmodium falciparum* infections in children from a holoendemic area in Ghana, West Africa. *Tropical Medicine and International Health* **11**, 613–619.

Lavazec, C., Sanyal, S. and Templeton, T. J. (2006). Hypervariability within the *Rifin*, *Stevor* and Pfmc-2TM superfamilies in *Plasmodium falciparum*. *Nucleic Acids Research* **34**, 6696–6707.

Lavazec, C., Sanyal, S. and Templeton, T. J. (2007). Expression switching in the stevor and Pfmc-2TM superfamilies in *Plasmodium falciparum*. *Molecular Microbiology* **64**, 1621–1634.

Le Roch, K. G., Zhou, Y., Blair, P. L., Grainger, M., Moch, J. K., Haynes, J. D., De La Vega, P., Holder, A. A., Batalov, S., Carucci, D. J. and Winzeler, E. A. (2003). Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301**, 1503–1508.

Lindenthal, C., Kremsner, P. G. and Klinkert, M.-Q. (2003). Commonly recognised *Plasmodium falciparum* parasites cause cerebral malaria. *Parasitology Research* **91**, 363–368.

Ljungström, I., Perlmann, H., Schlichtherle, M., Scherf, A. and Wahlgren, A. (2004). *Methods in Malaria Research*. Malaria Research and Reference Reagent Resource Center (MR4).

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press

Llinas, M., Bozdech, Z., Wong, E. D., Adai, A. T. and DeRisi, J. L. (2006). Comparative whole genome transcriptome analysis of three *Plasmodium falciparum* strains. *Nucleic Acids Research* **34**, 1166–1173.

McRobert, L., Preiser, P., Sharp, S., Jarra, W., Kaviratne, M., Taylor, M. C., Renia, L. and Sutherland, C. J. (2004). Distinct trafficking and localization of STEVOR proteins in three stages of the *Plasmodium falciparum* life cycle. *Infection and Immunity* **72**, 6597–6602.

Molineaux, L. and Gramiccia, G. (1980). *The Garki Project*, World Health Organization, Geneva, Switzerland.

Newton, P., Suputtamongkol, Y., Teja-Isavadharm, P., Pukrittayakamee, S., Navaratnam, V., Bates, I. and White, N. (2000). Antimalarial bioavailability and disposition of artesunate in acute *falciparum* malaria. *Antimicrobial Agents and Chemotherapy* **44**, 972–977.

Ofori, M. F., Dodoo, D., Staalsoe, T., Kurtzhals, J. A., Koram, K., Theander, T. G., Akanmori, B. D. and Hviid, L. (2002). Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens. *Infection and Immunity* **70**, 2982–2988.

Przyborski, J. M., Miller, S. K., Pfahler, J. M., Henrich, P. P., Rohrbach, P., Crabb, B. S. and Lanzer, M. (2005). Trafficking of STEVOR to the Maurer's clefts in *Plasmodium falciparum*-infected erythrocytes. *European Molecular Biology Organization* **24**, 2306–2317.

Recker, M., Nee, S., Bull, P. C., Kinyanjui, S., Marsh, K., Newbold, C. and Gupta, S. (2004). Transient cross-reactive immune responses can orchestrate antigenic variation in malaria. *Nature, London* **429**, 555–558.

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K. and Newbold, C. I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature, London* **357**, 689–692.

Schreiber, N., Brattig, N., Evans, J., Tsiri, A., Horstmann, R. D., May, J. and Klinkert, M.-Q. (2006). Cerebral malaria is associated with IgG2 and IgG4 antibody responses to recombinant *Plasmodium falciparum* RIFIN antigen. *Microbes and Infection* **8**, 1269–1276.

Sharp, S., Lavstsen, T., Fivelman, Q. L., Saeed, M., McRobert, L., Templeton, T. J., Jensen, A. T., Baker, D. A., Theander, T. G. and Sutherland, C. J. (2006). Programmed transcription of the *var* gene family, but not of *stevor*, in *Plasmodium falciparum* gametocytes. *Eukaryotic Cell* **5**, 1206–1214.

Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A. and Wellems, T. E. (1995). The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**, 89–100.

Sutherland, C. J. (2001). *Stevor* transcripts from *Plasmodium falciparum* gametocytes encode truncated polypeptides. *Molecular and Biochemical Parasitology* **113**, 331–335.

Winstanley, P., Edwards, G., Orme, M. and Breckenridge, A. (1987). The disposition of amodiaquine in man after oral administration. *British Journal of Clinical Pharmacology* **23**, 1–7.

https://doi.org/10.1017/S0031182007003794 Published online by Cambridge University Press