INFECTION AND IMMUNITY, Dec. 2007, p. 5967–5973 Vol. 75, No. 12
0019-9567/07/$08.00+0 doi:10.1128/IAI.00327-07

Characterization of the Antibody Response against *Plasmodium falciparum* Erythrocyte Membrane Protein 1 in Human Volunteers▽†

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Received 1 March 2007/Returned for modification 27 April 2007/Accepted 4 September 2007

The immune response against the *Plasmodium falciparum* variant surface antigen *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a key component of clinical immunity against falciparum malaria. In this study, we used sera from human volunteers who had been infected with the *P. falciparum* 3D7 strain to investigate the development, specificity, and dynamics of anti-PfEMP1 antibodies measured against six different strain 3D7 Duffy binding-like domain 1α (DBL1α) fusion proteins. We observed that a parasitemia of 20 to 200 infected erythrocytes per μl was required to trigger an antibody response to DBL1α and that antibodies against one DBL1α variant cross-react with other DBL1α variants. Both serum and purified immunoglobulin Gs (IgGs) were able to agglutinate infected erythrocytes, and purified anti-DBL1α IgGs bound to the live infected red blood cell surface in a punctate surface pattern, confirming that the IgGs recognize native PfEMP1. Analysis of sera from tourists naturally infected with *P. falciparum* suggests that the anti-PfEMP1 antibodies often persisted for more than 100 days after a single infection. These results help to further our understanding of the development of acquired immunity to *P. falciparum* infections.

Children living in regions of Africa where malaria is endemic experience decreasing numbers of clinical malaria episodes with increasing age (4, 30, 31), indicating the development of acquired immunity against malaria. The parasite protein *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is thought to be essential for the development of acquired clinical immunity to falciparum malaria (7, 12, 22) since agglutinating antibodies, mostly against PfEMP1, correlate with clinical protection against disease (7, 22).

PfEMP1 is expressed on the surface of infected red blood cells (IRBCs) and has been shown to mediate adherence to a range of host receptors located on the endothelial lining of specific organs and on uninfected RBCs (1, 2, 5, 16, 39, 40, 47, 48). PfEMP1 is encoded by a family of *var* genes, with each parasite genome containing approximately 60 different *var* genes (19, 45). Based on chromosomal location, gene orientation, and the 5′ flanking sequences in *P. falciparum* strain 3D7, *var* genes have been grouped into five distinct groups commonly called A, B, C, D, and E (19, 26, 49), with two possible intermediate groups (B/A and B/C) (28). PfEMP1 undergoes antigenic variation (41) caused by a switch in transcription between *var* genes. Each PfEMP1 molecule consists of a variable number of structurally unique domains. There are three types of domains: DBL, CIDR, and C2. Within the DBL category, there are six sequence classes (DBL-α, -β, -γ, -δ, -ε, and -X), while there are only two CIDR sequence classes (CIDR-α and CIDR-β), and the C2 domain is conserved (19). While the tertiary structure of PfEMP1 is still to be elucidated, the different domains appear to have conserved but different functions: CIDR-α binds CD36 (2, 42), DBL-γ binds to chondroitin sulfate A (5, 18, 37), and DBL-α is involved in rosetting (8, 40).

The diversity of the PfEMP1 repertoire of parasites in a given geographic area is a key factor in the development of clinical immunity. Other factors that may also be important in determining the development and maintenance of clinical immunity are (i) the parasite density required to trigger an anti-PfEMP1 antibody response, (ii) the specificity and affinity of the anti-PfEMP1 immune responses, and (iii) the longevity of these antibodies. At present, none of these factors is well defined.

In a setting with endemic disease and with high parasite diversity, many infections may be needed to develop clinical immunity. However, it appears that an immune response against a specific parasite may start to develop after a single infection (10). Convalescent-phase sera collected from patients living in regions of endemicity appear to have some cross-reactivity since the antibodies agglutinate not only the parasite isolate infecting the patient but also other parasite isolates (7, 20). However, it is unclear from these studies if this cross-reactivity is due to multiple reactivities of specific antibodies or previous exposure to the same (or highly similar) parasites boosting a memory response. In the absence of reexposure to

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 8 October 2007.

TABLE 1. Summary of infection characteristics and antibody responses in human volunteers[a]

Volunteer(s) (reference[b])	Results for first 3D7 infection				Results for second or other 3D7 infection			
	Peak parasitemia (IRBC/μl)	Serum sample collection time(s)[c]	Titer at which response occurred to:[d]		Peak parasitemia (IRBC/μl)	Serum sample collection time(s)[c]	Titer at which response occurred to:[d]	
			IgM	IgG			IgM	IgG
B1 (9)	18	Pre, 26, 42	Neg	Neg	~1	Pre, 16	Neg, 1/200	Neg, Neg
B2 (9)	210	Pre, 18, 41	Neg, 1/900, 1/900	Neg, Neg, 1/900	~1	Pre, 14, 46	Neg, 1/900, 1/900	Neg, 1/900, 1/900
C3 and C4 (29)	~1	14	Neg	Neg	−	−	−	−
C5–C19 (29)	≤10	≤40	Neg	Neg	−	−	−	−
C20 (29)	8	32	1/200	Neg	−	−	−	−
C21 (29)	10	34	1/200	Neg	−	−	−	−
D1	5,000	14, 60	1/600, Neg	Neg, Neg	−	−	−	−
G1–G4 (36)	<1	NA	NA	NA	<1	40[e]	−	Neg
E1 and E2	<10	NA	NA	NA	<10	14[f]	Neg	Neg

[a] Neg, no detectable antibodies; −, only infected once; NA; not available.
[b] Studies describing the infection protocol for volunteers are indicated where such information has been published.
[c] Pre indicates sample collected preinfection. Numbers indicate days postinfection (number of days excluding liver stages) of sample collection.
[d] Antibody responses were measured against PF11_0007DBL1α fusion protein using Western blotting.
[e] Serum sample taken 40 days after the fourth infection.
[f] Serum sample taken 14 days after the third infection.

P. falciparum, antibody levels decline over time, and the longevity of protective immune responses has been disputed, with both short-lived (17, 35) and long-term responses being reported (11, 13).

In an effort to better define these key factors, we have used a collection of serum and plasma samples stored from several previous vaccine studies to investigate the development, specificity, and longevity of anti-Duffy binding-like domain 1α (DBL1α) antibodies in well-monitored *P. falciparum* infections. The DBL1α domain of PfEMP1 was selected for analysis since antibodies directed against variable epitopes of this region correlate with the level of exposure to malaria (33), suggesting that it may play a role in clinical immunity. The results indicate that anti-PfEMP1 antibodies can develop after a single infection, provided the parasitemia is sufficient. Additionally, these antibodies cross-react with other PfEMP1 variants in both denatured and native conditions, and PfEMP1 antibodies can persist for at least as long as antibodies to other malaria antigens. These results provide a unique insight into anti-PfEMP1 antibody production after limited exposure to the parasite.

MATERIALS AND METHODS

Sera from volunteers. Stored serum/plasma samples from 27 volunteers involved with previous *P. falciparum* strain 3D7 infection studies (9, 29, 36) and from one laboratory-infected individual (D1) were used in this study (Table 1). All serum/plasma samples were stored at −80°C. Ethics approval for the use of these stored samples was granted by the QIMR human research ethics committee.

Sera from tourists. Serum samples from Australian residents who contracted falciparum malaria overseas but became symptomatic upon returning to Australia were collected at the Royal Brisbane and Princess Alexandra Hospitals in Brisbane, Australia (14), and used in this study. Serum samples were taken at the time of treatment and several months thereafter.

Negative and positive control sera. Sera from healthy anonymous blood donors were obtained from the Australian Red Cross blood service. Pools of 10 to 12 individual sera were used as negative controls. The positive control was a stored serum sample from a hyperimmune individual living in Papua New Guinea.

Expression and purification of recombinant DBL1α. Recombinant fusion proteins encoded by the DBL1α domains of six *var* genes with different structures and genomic locations (26, 28) were expressed in *Escherichia coli*. The genes selected also had different transcript abundances in parasite samples (3D7B1 and 3D7B2) from two of the infected volunteers (34). Four of the DBL1α domains were from group B *var* genes: PFC0005w, PF11_0007, MAL6P1.1, and PF10_0406 had been detected as major transcripts, corresponding to AFBR13, -16, -28, and -41, respectively, in the 3D7B1 and 3D7B2 samples (34). The remaining two fusion proteins were from group A (PF13_0003 [AFBRNT1]) and group C (PF07_0051 [AFBRNTL]), and neither was a dominant transcript in either 3D7B1 or 3D7B2.

The DBL1α regions of the selected variants were PCR amplified using gene-specific primers (see Table S1 in the supplemental material) and cloned into the TOPO vector pET160 (Invitrogen, CA). The recombinant plasmid was transfected into BL21Gold *E. coli* cells and induced to express protein with 0.1 mM isopropyl-β-D-thiogalactopyranoside in a total volume of 400 ml LB medium for 4 h. The *E. coli* cells were pelleted, washed in phosphate-buffered saline (PBS), and frozen at −70°C overnight before being sonicated (1 min of pulsing two times) in guanidinium lysis buffer (6 M guanidinium hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM sodium chloride) at 4°C. The lysate was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant was applied to washed nickel-agarose beads (Invitrogen, CA) for affinity binding. After the binding, the beads were put into a column and washed with buffer A (20 mM sodium phosphate, 6 M urea, 500 mM sodium chloride, 1% Triton X-100, 1 mM mercaptoethanol, pH 7.8). For on-column refolding, a linear gradient of a decreasing concentration of urea was applied to the 2-ml column over 40 min at a flow rate of 1.5 ml/min using increasing amounts of buffer B (20 mM sodium phosphate, 500 mM sodium chloride, 1% Triton X-100, 1 mM mercaptoethanol, pH 7.8). After the column was washed with 10 volumes of buffer B, the refolded fusion proteins were eluted from the nickel-agarose using 10 ml of 1 M imidazole (pH 7.8). The proteins were dialyzed, concentrated using iCON concentrators (Pierce, IL), and boiled in sodium dodecyl sulfate sample buffer before being loaded onto a 12% polyacrylamide gel electrophoresis gel for protein separation.

Parasite protein extraction and anti-PfEMP1 antibodies. Strain 3D7-infected RBCs (3D7-IRBCs) were cultured in vitro under standard conditions (46), sorbitol synchronized (27), harvested at late trophozoite/early schizont stages, and extracted as previously described (44). To extract full-length native PfEMP1, we followed the method of Beeson et al. (3) and used 3D7B2 parasites which had been cultured for 23 days or the same number of uninfected RBCs as the control.

Western blot analysis. The recombinant protein and the parasite protein extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were cut into strips and blocked with 5% skim milk in PBS–0.05% Tween 20 (5% MPBST) for 1 h. The strips were incubated with plasma/serum samples diluted 1/200, 1/600, 1/900, or 1/2,700 in 5% MPBST for at least 1 h at room temperature. After being washed three times with PBST, the membranes were incubated with 1/2,000 horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) or IgM and washed three times in PBST. The rabbit anti-acidic terminal segment (anti-ATS) antibody directed against the conserved cytoplasmic region of the PfEMP1 protein was kindly provided by Stephen Rogerson and was used at a 1 in 200 dilution in 5% MPBST. Signals were developed using enhanced

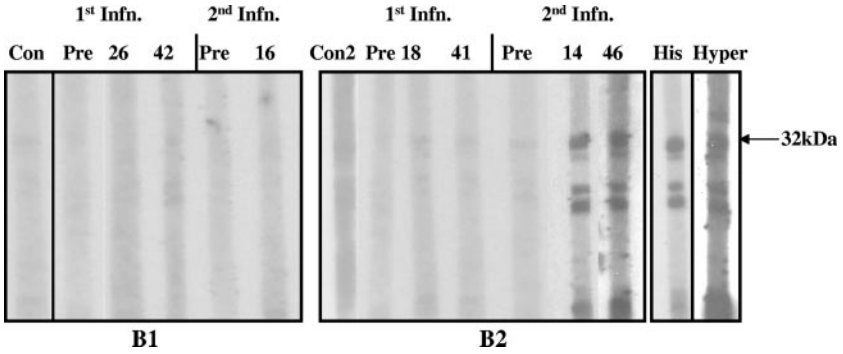

FIG. 1. IgG antibody reactivity to PF11_0007DBL1α in sera from volunteers B1 and B2 collected prior to (Pre) and at the indicated number of days after the first and second infections (Infn.), excluding the liver stages. Con1 and Con2 represent two different pools of Red Cross sera used as negative controls. Hyper, hyperimmune sera used as a positive control. His, anti-His antibody used to reveal protein loadings. A molecular size marker is shown on the right.

chemiluminescence (Amersham Bioscience) and captured on autoradiography film.

Enzyme-linked immunosorbent assays (ELISAs). Ninety-six-well plates were coated with 1 μg/well (in 100 μl PBS) of refolded recombinant DBL1α overnight at room temperature, washed three times for 1 min in PBS, and blocked in 5% MPBST for 1 h. Sera (diluted 1/100 to 1/1,000 in 5% MPBST) were added to wells and incubated for 1 h. After the wells were washed with 2.5% MPBST (three times for 3 min), horseradish peroxidase-conjugated goat anti-human IgG (1/2,000 dilution in 5% MPBST) was added to the wells and incubated for 1 h. The plate was washed three times with 2.5% MPBST, developed using TMB One solution (Promega), and then read at 415 nm.

Live cell imaging. Parasites originating from volunteer B2 were cultured in intact erythrocytes for 23 days to mid-stage pigmented trophozoites prior to analysis. The cells were washed three times in PBS supplemented with 1% bovine serum albumin (PBSA) and centrifuged for 3 min at 140 × *g*. The cells were resuspended to a 7.5% hematocrit (5×10^8 cells/ml). Two microliters of purified IgG from volunteer B2 (human anti-PF11_0007DBL1α) was added to 25 μl of cells and incubated at 37°C for 1 h with gentle mixing every 30 min. The cells were then washed three times in PBSA (3 min at 140 × *g*) and incubated for 1 h with rabbit anti-human IgG at a dilution of 1/50 in PBSA. The cells were again washed three times as described above and then incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (DAKO) diluted 1/50 in PBSA for 1 h at 37°C. The cells were again washed three times in PBSA. Samples were viewed with an inverted Leica TCS0SP2 confocal microscope using 100× oil immersion objectives (numerical aperture, 1.4). A helium-neon laser (543-nm line) equipped with the appropriate dichroics was used for excitation.

Purification of DBL1α variant-specific IgGs. Two recombinant fusion proteins, PF11_0007DBL1α and PF07_0051DBL1α, were cross-linked to separate columns using a Sulfolink kit (Pierce, IL). Serum from volunteer B2 was loaded onto the protein columns, rocked gently for 1 h at room temperature, and then washed with PBS to remove unbound IgGs. Bound IgGs were eluted using 10 ml of 100 mM glycine (pH 4.5), followed by 10 ml of 100 mM glycine (pH 2.5). The 1-ml eluted IgG fractions were neutralized using 1 M Tris (pH 7.2) and used for Western blot analysis.

Agglutination. IRBCs from volunteers B1 and B2 which were collected at the time of treatment of the first infection were thawed and cultured in vitro for 21 days. These cultures, along with IRBCs from long-term cultures of strain 3D7, were resuspended at a 5% hematocrit in RPMI 1640 containing ethidium bromide (final concentration of 10 μg/ml). Amounts of 20 μl of the cell suspensions were incubated with 5 μl of control serum, sera from individuals who had no IgGs against DBL1α fusion proteins (B1, C3, C4, and G1), serum from volunteer B2, or IgG purified from serum from volunteer B2. Reactions were conducted in round-bottomed 96-well plates for 60 min with gentle rotation at room temperature. The control serum plus the four samples with nondetectable anti-DBL1α IgGs were used as negative controls. To score the agglutination, cells were examined using wide-field fluorescence microscopy (40× objective), with 25 fields being counted. Only agglutinates of more than three IRBCs were counted and scored (6).

Disruption of rosette formation in *P. falciparum* R29. The R29 parasite line which had been selected for rosette formation (38) was used for rosette disruption analysis. The R29 trophozoite- or schizont-infected erythrocytes were incubated for 1 h at 37°C in the presence of serum from volunteer B2, as well as four different controls (blood bank serum and three other sera which had no anti-DBL1α IgGs), at a 1/10 dilution. Dextran sulfate (0.1 mg/ml) was used as a disruption positive control. Three rosette disruption experiments were conducted, each in triplicate.

Statistical analysis. Differences in the peak parasitemia between individuals with and without a positive IgM response against recombinant DBL1α were analyzed using the Mann-Whitney test. Binary logistic regression was used to estimate the parasitemia required to produce a positive IgM response after one infection.

RESULTS

Antibodies against the DBL1α region were detected in volunteers with parasitemia above a threshold. The DBL1α regions' six PfEMP1 variants were expressed in *E. coli*, purified to near homogeneity, and used to screen for any immunoreactive IgM and IgGs in sera collected from 28 individuals (Table 1). After a single infection, two individuals, volunteers B2 and D1, developed IgM responses with titers of 1/900 and 1/600, respectively, while two other volunteers (C20 and C21) developed weak but detectable IgM responses at a titer of 1/200 to PF11_0007DBL1α. The peak parasitemia experienced by this group of four individuals was significantly higher than that in the remaining 24 volunteers who did not develop an IgM response ($P < 0.01$). The level of parasitemia that best distinguished between individuals with or without a positive IgM response was 19.4 infected erythrocytes/μl ($P < 0.001$; Nagelkerke $R^2 = 0.558$).

Eight individuals experienced a second infection. Following the second infection, volunteer B1 developed a weak IgM response (titer of 1/200) to PF11_0007DBL1α but did not show any IgG reactivity against any of the six fusion proteins (Table 1). Volunteer B2 developed a significant IgG response to PF11_0007DBL1α fusion protein, with a titer of 1/900 (Table 1 and Fig. 1), and a similar response to the other five fusion proteins (data not shown). The remaining six volunteers did not develop a detectable IgM response to PF11_0007DBL1α or an IgG response to any of the six fusion proteins after this and further infections (Table 1). The IgG response to DBL1α in serum from volunteer B2 was confirmed by a positive response in an ELISA plate assay using refolded PF11_0007 DBL1α (data not shown).

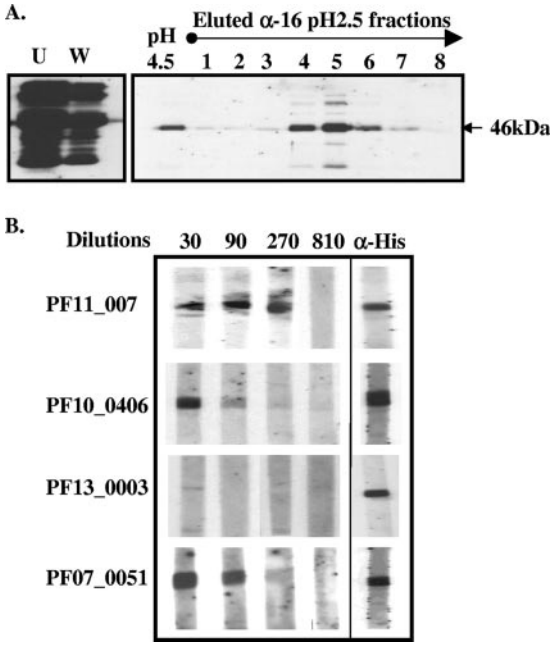

FIG. 2. Reactivities of IgGs, purified on a PF11_0007 affinity column, to DBL1α fusion proteins. (A) Human IgG was detected in the following fractions by Western blotting using anti-human IgG: U, unbound IgG; W, wash; pH 4.5 elution; and pH 2.5 elution (lanes 1 to 8) from the PF11_0007DBL1α column. A molecular size marker is shown on the right. (B) Reactivity of serially diluted IgG in fraction 5 of the pH 2.5 elution to fusion proteins PF11_0007DBL1α, PF10_0406DBL1α, PF13_0003DBL1α, and PF07_0051DBL1α. α-His, anti-His antibody.

Antibodies against one DBL1α variant cross-react with other DBL1α variants. To assess the affinity and specificity of the anti-DBL1α response in serum from volunteer B2, IgGs against one specific variant, PF11_0007DBL1α, were affinity purified on the refolded recombinant of this variant and tested for reactivity to it and the other five variants. The purified anti-PF11_0007DBL1α IgGs (eluted from the column at pH 2.5) reacted with the PF11-0007DBL1α fusion protein (group B) at dilutions of 1/30, 1/90, and 1/270; with PF07_0051DBL1α (group C) at dilutions of 1/30 and 1/90; and with PF10_0406DBL1α (group B) at a 1/30 dilution only (Fig. 2). The IgGs failed to recognize PF13_0003DBL1α (group A) (Fig. 2). The purified IgGs also reacted with recombinant proteins of PFC0005wDBL1α (group B) and MAL6P1.1DBL1α (group B) at 1/90 dilution (data not shown). This indicates that IgGs purified on the PF11_0007DBL1α column had a high affinity to the PF11_0007DBL1α fusion protein and cross-reacted with the fusion proteins of other variants from groups B and C, albeit at lower dilutions.

IgGs against a second fusion protein, PF07_0051DBL1α (group C), were also purified from serum from volunteer B2 and tested in the same manner. The purified IgGs reacted with all fusion proteins tested; however, the response to the PF07_0051DBL1α fusion protein was much stronger than to the other five fusion proteins (data not shown). The hyperimmune serum was strongly reactive towards 3D7 DBL1α at a 1/300 dilution (Fig. 1) and possessed a titer of 1/900, which was the same as that of the serum from volunteer B2.

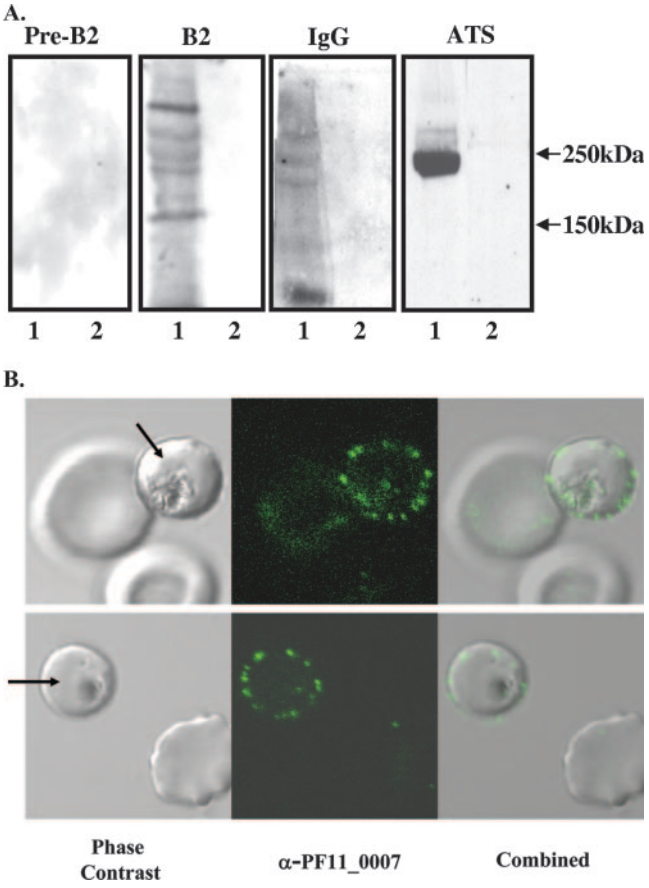

FIG. 3. Serum from volunteer B2 and purified IgGs recognize full-length PfEMP1 on Western blot of parasite extract and on the live IRBC surface. (A) A Western blot of 3D7B2-IRBCs (day 23 in culture) was probed with preimmune serum from volunteer B2 (Pre-B2), postinfection serum from volunteer B2 (B2), or IgGs purified on the PF11_0007DBL1α column. Anti-ATS antibody was used as a positive control for PfEMP1, as it is a rabbit antibody against the cytoplasmic C-terminal ATS region of PfEMP1. For each blot, lane 1 represents 3D7B2 extract and lane 2 represents extract from the same number of uninfected RBCs. Molecular size markers are shown on the right. (B) Two images of live, intact 3D7-IRBCs surface labeled with IgG from volunteer B2 purified on the PF11_0007DBL1α column. The three panels show a differential interference contrast image, an immunofluorescence image, and the merged image. The arrows point to *Plasmodium falciparum*-infected RBCs.

Reactivities of volunteers' sera against malaria proteins. Serum and purified IgGs from volunteer B2 after the second infection recognized several proteins of more than 200 kDa in extracts from 3D7B2 parasites that had been cultured in vitro for 23 days (Fig. 3). An anti-ATS antibody recognized a broad band of a similar molecular weight, which is expected for full-length PfEMP1. These results indicate that IgGs from volunteer B2 may recognize native PfEMP1. Serum from volunteer B2 also appears to recognize other high-molecular-weight parasite proteins. Sera from volunteer B1 after boosting and from volunteer B2 after the first infection did not have any detectable IgG response to malarial proteins of more than 200 kDa but recognized several bands of less than 200 kDa.

Serum from volunteer B2 and purified anti-DBL1α IgGs agglutinate IRBCs and recognize surface PfEMP1. Agglutina-

tion assays were performed to test whether the detected antibodies recognize native proteins. Serum from volunteer B2 collected 21 days after the second infection agglutinated parasites that caused the first infection in this volunteer and also agglutinated parasites originating from the first infection of volunteer B1. However, the same serum from volunteer B2 failed to agglutinate long-term-culture strain 3D7 parasites. Control sera and preinfection and post-first-infection sera from volunteer B2, as well as postinfection sera from volunteer B1, did not agglutinate IRBCs from either volunteer B1 or volunteer B2 or the long-term-cultured 3D7. IgGs purified against both PF11_0007DBL1α and PF07_0051DBL1α fusion proteins were able to agglutinate parasites originating from both volunteers but not parasites from the long-term culture. Sera from volunteers C3, C4, and G1 were also not able to agglutinate any of the 3D7-IRBCs tested (data not shown). These results indicate that the ability of serum from volunteer B2 to recognize DBL1α on a Western blot mirrors its ability to agglutinate 3D7B2-IRBCs.

Further evidence that IgGs from volunteer B2 can recognize native PfEMP1 was obtained by surface labeling 3D7B2-IRBCs with purified IgGs from serum from volunteer B2. Figure 3B shows that IgGs eluted from the PF11_0007DBL1α column labeled the surface of intact IRBCs in a punctate ring pattern, as reported for other antibodies that recognize surface-exposed epitopes on PfEMP1 (15). Uninfected RBCs did not show any staining with the IgGs from volunteer B2 (Fig. 3B).

Serum from volunteer B2 inhibits rosetting. We also tested the abilities of sera from volunteers to disrupt the rosette formation of strain R29. In the presence of control sera, 30% ± 4.6% (mean ± standard deviation) of R29-IRBCs bound two or more RBCs. This value was reduced to 6% ± 1.5% when dextran sulfate or post-second-infection serum from volunteer B2 was added to the culture. No reduction in rosette formation was observed when control or preinfection serum from volunteer B1 or volunteer B2 was tested.

3D7 DBL1α variants were recognized by sera from tourists infected with *P. falciparum*. To further characterize the cross-reactivity of human IgGs against DBL1α and analyze the longevity of the response, we assessed the immune reactivities to the 3D7 DBL1α fusion proteins of serum samples from 19 tourists. Sera from 8 of the 19 tourists (42%) showed reactivity against PF10_0406DBL1α, PF11_0007DBL1α, and MAL6P1.1DBL1α in a Western blot assay (data not shown), suggesting the presence of cross-reactive antibodies in these individuals. Five of these eight tourists had no previous *P. falciparum* infection. However, tourist gg had lived in a country in which malaria was endemic. Convalescent-phase serum samples taken from these five patients 31 to 281 days postdiagnosis had detectable antibody responses to the 3D7 DBL1α fusion protein (Table 2). Only one convalescent-phase serum sample returned a negative result; tourist qq converted from being reactive on day 31 to negative on day 143 postdiagnosis (Table 2).

TABLE 2. Longevity of reactivities of antibodies from travelers infected with *P. falciparum* against strain 3D7 DBL1α fusion proteins

Patient	Parasitemia at diagnosis (IRBC/μl)	Infection acquired in:	Serum sample collection time(s)[a]	Reactivity[b]
aa	326,000	Thailand	91	+
cc	14,484	Papua New Guinea	281	+
gg	7,040	Pakistan	176	+
mm	2,600	Papua New Guinea	187	+
qq	215,000	Papua New Guinea	31, 143	+, −

[a] Days postdiagnosis.
[b] Reactivity of convalescent-phase serum to 3D7 DBL1α. Reactivity was positive on day 31 postdiagnosis and negative on day 143 postdiagnosis.

DISCUSSION

The host immune response against PfEMP1 is a key component in the development of clinical immunity to falciparum malaria (7, 12, 22). However, the diversity of this protein within and between parasites, combined with antigenic switching between variants, makes studying the development of PfEMP1-specific immunity difficult. We have investigated three key parameters defining the IgG immune response to PfEMP1: the trigger (parasite biomass) required to generate a response, the cross-reactivities of anti-PfEMP1 antibodies, and the longevity of the antibodies. Many of the experimental results presented are based on data from Western blot analysis which primarily measures IgM or IgG reactivity to denatured proteins. We chose Western blot analysis over ELISA because Western blot analysis can discriminate for reactivity against contaminating *E. coli* proteins. We subsequently demonstrated that antibodies against fusion proteins were able to recognize native PfEMP1, as indicated by the agglutination of parasites expressing native PfEMP1 and live-cell staining of 3D7B2-IRBCs.

The trigger for an immune response to PfEMP1 is dependent on the overall parasite biomass and the proportions of parasites expressing different PfEMP1 variants. In this study, serum samples from 28 individuals who were infected with *P. falciparum* strain 3D7 parasites were analyzed and their natural progression of infection was monitored. Therefore, this study provides a unique opportunity to assess the development of immune responses to PfEMP1. Of the samples tested, an anti-DBL1α IgM response was detected in samples from four individuals after a single infection. As a group, these volunteers had significantly higher peripheral parasitemia than the volunteers who did not develop an anti-DBL1α IgM response. One of these four volunteers (volunteer B2) developed a significant anti-DBL1α IgG response after a second infection, suggesting that the response was triggered during the first infection and boosted upon reexposure. In contrast, the five volunteers (B1, G1, G2, G3, and G4) who did not have detectable IgM responses after the first infection but received subsequent infections did not develop a detectable anti-DBL1α IgG response, indicating that the response was not triggered during the first infection. The results suggest that the presence of an IgM response is an indicator for the trigger of an IgG response.

We hypothesize that the parasite threshold required to stimulate an IgG response to PfEMP1 in 3D7 parasites is between 20 and 200 parasites/μl, based on the peak parasitemia during the first infection in volunteers who were infected on multiple occasions and on the detection of an IgM response. The actual number of parasites expressing a given PfEMP1 variant that are required to stimulate a response to that variant is unknown, as we were not able to determine the actual number of

parasites expressing each variant. Parasitemias below this threshold did not appear to trigger an anti-PfEMP1 IgG response even after repeated infection with the same parasites. In contrast, IgG antibodies to other falciparum proteins were detected in volunteers who did not develop an anti-DBL1α IgG response, suggesting a lower trigger for other parasite proteins. This difference may be due to differences in the antigenicities of various proteins and to the fact that only a subpopulation of parasites express a specific PfEMP1 variant.

Immune responses developed against parasite surface antigens, including PfEMP1, have been shown to correlate with protection against malaria infections in a variant-specific manner (7, 32). In this study, the antibodies generated by volunteer B2 were able to detect multiple forms of DBL1α. While specific anti-DBL1α antibodies to multiple variants are likely to have developed due to exposure to various PfEMP1, we demonstrated that several DBL1α fusion proteins were recognized by antibodies purified on the PF11_0007DBL1α and PF07_0051 DBL1α columns. This cross-reactivity could be due to the sequence and structural homology between the variant DBL1α domains. Overall, this observation, combined with the fact that 42% of tourist serum samples recognized 3D7 DBL1α, suggests the existence of antibody cross-reactivity.

While reports from field studies indicate the presence of cross-reactive antibodies against PfEMP1 (7, 24), this is the first time that cross-reactivity has been reported for variant-specific IgGs after well-defined clinical infections. Further evidence of cross-reactivity came from the rosette disruption experiment. The serum from volunteer B2 disrupted rosette formation in the R29 *P. falciparum* strain. These results indicate that there is cross-reactivity between different PfEMP1 variants within the same parasite and between different parasites as opposed to reactivity resulting from previous exposures. The results also suggest that antibodies developed against one *P. falciparum* strain may have some functionality against different parasite lines. This is consistent with the recent report that a single *P. falciparum* infection is sufficient to induce antibodies reactive to PfEMP1 of heterologous strains (14). However, whether the cross-reactivity is sufficient and has the longevity to offer protection against heterologous infections or influences the number of exposures required to induce clinical immunity remains to be elucidated.

It has been proposed that the maintenance of antibody responses to malaria is dependent on frequent or persistent infection (23), with agglutinating antibodies reported to last from a few months to a year in the absence of boosting (21, 23). We investigated the longevity of anti-DBL1α antibodies using convalescent-phase sera from tourists. Since it is highly unlikely that any of the tourists were infected with the 3D7 parasite strain, our analysis was based on detecting cross-reactive antibodies. Even so, the majority of tourists had detectable antibodies for more than 100 days, consistent with the duration reported in Kenyan children (25) and suggesting that anti-PfEMP1 antibodies persist longer than IgGs against merozoite surface proteins (43).

This unique study has shown that an antibody response to the DBL1α domain of PfEMP1 can be triggered by a parasitemia above 20/μl and can be boosted significantly by exposure to the same parasite at a much lower parasitemia; an antibody class switch from IgM to IgG accompanies this boosting. We have demonstrated that antibodies against one PfEMP1 variant cross-react with other variants and that the levels of cross-reactivity may differ between certain groupings of PfEMP1. These antibodies agglutinate parasite-infected erythrocytes and bind to the surface of IRBCs and disrupt rosette formation and, therefore, may play an important role in modulating the clinical presentation of malaria and its potential to develop severe complications.

ACKNOWLEDGMENTS

The project was funded by an NIH grant (5R01AI047500-04/05) and Department of Defense, Australia. D.R.K. also received a travel grant from the Australian ARC/NHMRC parasitology network.

We thank Chris Ockenhouse for providing sera from mosquito-infected volunteers, David Roberts for providing R29 parasites, Anthony Stowers for advice on protein expression, Allan Saul for constructive discussion, and Dennis Shanks, Katharine Trenholm, James McCarthy, and Michelle Wykes for critical review of the manuscript. We acknowledge Kathy Davern, Tim Byrne, and Graham Brown for providing the rabbit anti-ATS antibody, and we also thank Nanhua Chen for parasite culture and Jennifer Peters for sequence alignment and database searches. We thank the Australian Red Cross blood services, Brisbane, for providing the human red blood cells and sera used in this study.

The opinions expressed herein are those of the authors and do not necessarily reflect those of the Defense Health Service or any extant policy of Department of Defense, Australia.

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Editor: J. F. Urban, Jr.