Overlapping antigenic repertoires of variant antigens expressed on the surface of erythrocytes infected by *Plasmodium falciparum*

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SUMMARY

Antibodies against variable antigens expressed on the surface of *Plasmodium falciparum*-infected erythrocytes are believed to be important for protection against malaria. A target for these antibodies is the *P. falciparum* erythrocyte membrane protein 1, PfEMP1, which is encoded by around 50 *var* genes and undergoes clonal variation. Using agglutination and mixed agglutination tests and flow cytometry to analyse the recognition of variant antigens on parasitized erythrocytes by plasma antibodies from individuals living in Daraweesh in eastern Sudan, an area of seasonal and unstable malaria transmission, we show that these antibodies recognize different variant antigens expressed by parasites of different genotype. Comparing the levels and acquisition of antibody to variant antigens in pairs of parasite isolates expressing different variant types, there is a correlation between the acquisition of antibodies to some combinations of variant antigens but not to others. These results indicate that (1) a single infection will induce the production of antibodies recognizing several variants of surface-expressed antigens, (2) the repertoire of variable antigens expressed by different parasites is overlapping and the degree of overlap differs between isolates, and (3) the expression of at least some variant antigens is genetically linked.

Key words: *Plasmodium falciparum*, PfEMP1, variant antigens, unstable malaria.

INTRODUCTION

Individuals living in areas of stable malaria transmission develop immunity to malaria over a period of years (Christophers, 1924). The immunological effector mechanisms responsible for protection have not been clearly defined, but the presence of antibodies agglutinating erythrocytes infected with late blood-stage parasites has repeatedly been linked to development of protective immunity (Marsh *et al*. 1989). The main target for agglutinating antibodies is believed to be *P. falciparum* erythrocyte membrane protein 1, PfEMP1 (Baruch *et al*. 1995). PfEMP1 is encoded by a large family of *var* genes (Su *et al*. 1995), and parasite clones can switch between expression of different PfEMP1 variants (Biggs *et al*. 1991; Roberts *et al*. 1992). PfEMP1 is responsible for adhesion of parasites to the endothelium of post-capillary venules (Magowan *et al*. 1988; Biggs *et al*. 1992) and is a ligand for the formation of rosettes between infected and uninfected erythrocytes (Rowe *et al*. 1997). Antibodies against PfEMP1 are believed to mediate protection against malaria through interfering with the binding of late blood-stage parasites to endothelium, a process that allows the parasites to escape removal in the spleen and liver (David *et al*. 1983).

In the village of Daraweesh in eastern Sudan, we have observed that plasma from a single individual usually agglutinates several of the parasite samples taken from different patients in the area, cryopreserved and then revived in *in vitro* culture (Giha *et al*. 1998). This finding implies that challenge with one infectious inoculum results in the formation of antibodies in the infected host that can cross-react with parasites infecting other individuals in the village at around the same time. In the present study, we have used a mixed agglutination assay and an assay to measure antibodies bound to the surface of infected erythrocytes by flow cytometry to show that although parasites circulating in the area are genetically diverse and express different variants of PfEMP1, they also have a considerable overlap in their PfEMP1 repertoires.

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MATERIALS AND METHODS

Study area

The study was conducted in Daraweesh, a village of 120 thatched mud huts situated 15 km south of the town of Gedaref, Sudan, and 450 km south-east of the capital Khartoum. The predominant malaria parasite species is *P. falciparum* (98 %) with *P. vivax* and *P. malariae* occasionally seen. Bed nets and chemoprophylaxis are not used. The area is meso-endemic for *P. falciparum* malaria, with seasonal and unstable transmission, usually peaking in October, following the annual rainy season. Little, if any, transmission occurs from January through July.

Study cohort and sampling strategy

This study is part of a longitudinal study of malaria in Daraweesh carried out since 1990. Blood samples were collected after informed consent from all available villagers before the malaria season in the beginning of September 1995 and again after the end of the season in January 1996. During the malaria season, all villagers were instructed to report any symptoms of malaria to the field team, which included a medical doctor, present in the village on a daily basis. From patients with symptoms or signs of malaria, slides were prepared and examined microscopically for the presence of *P. falciparum*. Patients were defined as having malaria and bled if they either reported fever or were febrile (temperature $> 37{\cdot}5$ °C) and were slide positive. During the season of 1995, 104 of the 421 villagers had at least 1 episode of malaria. Based on the availability of plasma samples, age and knowledge of whether the individuals had malaria during the season or not, 29 individuals were selected for this study. Sixteen of the individuals had experienced at least 1 clinical episode of malaria during the season. The individuals were between 5 and 58 years of age and 10 were males. The study received ethical clearance from the Ethical Committee of the University of Khartoum and national clearance from the Sudanese Ministry of Health.

Blood samples were drawn by venipuncture into heparinized vacutainers (Becton Dickinson, Rutherford, NJ, USA). Following centrifugation (200 ***g*** for 5 min), plasma was collected and stored frozen at 20 °C until used. From individuals with malaria, pelleted parasitized erythrocytes were washed 3 times in RPMI 1640 medium (Gibco, Paisley, UK), resuspended in 28 % glycerol cryopreserving medium, and stored in liquid nitrogen as described elsewhere (Merryman & Hornblower, 1972).

Parasite culture

Erythrocytes from 4 donors with malaria were thawed, washed and put into culture. The parasites were cultured according to standard procedures (Trager & Jensen, 1976) in O Rh^+ erythrocytes and RPMI supplemented by 5 % pooled Rh^+ human serum.

Parasite genotyping

The parasites were genotyped at the GLURP, MSP-1 and MSP-2 loci by PCR as described elsewhere (Roper *et al*. 1996, 1998).

Microagglutination assay

Antibody-mediated agglutination of parasitized erythrocytes was measured by modifications of previously published protocols (Sherwood *et al*. 1985; Marsh & Howard, 1986; Giha *et al*. 1998). Parasite cultures were propagated *in vitro* to parasitaemias of at least 2 %, the majority of the parasites being late trophozoites or schizonts. Cultures were then washed in phosphate-buffered saline (PBS) and the haematocrit adjusted to 20 %. Aliquots of erythrocyte pellet (50 μl), 25 ml of PBS containing 40 μg/ml of ethidium bromide, and 5 μl of test plasma were dispensed into 96-well microtitre plates (Nunc, Rødovre, Denmark). The plates were then sealed and incubated on a shaker (10 rpm) at 37 °C for 1 h. Subsequently, 1 μl samples were mounted on multispot slides under cover-slips and examined microscopically under visual and UV light. Agglutination, and the size of the largest agglutinates, were recorded and the degree of agglutination assessed on a 5-level semi-quantitative scale (Marsh & Howard, 1986): 0–3 parasites: 0, 4–9 parasites: +, 10–29 parasites: ++, 30–99 parasites: +++, 100 parasites or more: ++++.

Mixed agglutination assay

The ability of plasma to agglutinate 2 isolates of parasites was measured by a modification of the method developed by Newbold *et al*. (1992). The 2 isolates were incubated with either ethidium bromide (40 μg/ml) or acridine orange (0·01 mg/ml) for 30 min at room temperature. The erythrocyte pellet (25 ml) from each isolate, 25 μl of PBS and 5 μl test plasma were added to a microtitre plate. The plate was sealed, incubated on a shaker and samples scored as described above. The agglutinates were divided into those containing only parasites labelled with ethidium bromide, those containing acridine orange-labelled parasites only and those containing parasites labelled with both dyes. The 3 types of agglutinates were then scored as described above.

Flow cytometry

Cultures were grown to parasitaemia of 2–3 % with the majority of parasites in the trophozoite or schizont stages. Pelleted erythrocytes (500 μl) were

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Table 1. Genotypes of parasites causing disease in the 16 individuals who had malaria

(The parasites were typed by PCR targeting MSP-1 block 2, MSP-1 C-terminal, MSP-2 and GLURP.)

Isolate ID	Donor ID	MSP-1 Block 2	MSP-1 C-terminal	MSP-2	GLURP
Z361	J1	N.D.*	N.D.	N.D.	N.D.
Z370	C1	Mad 20	Wellcome	FC27	E
Z397	2R7	K1	Wellcome	FC27	F
Z403	A3	Ro33, K1	Wellcome	FC27, 3D7	K1, G
Z412	2H6	Ro33, K1, Mad 20	Mad 20, Wellcome	3D7, FC27	K, E
Z416	2J5	Mad 20	Wellcome	3D7	B
Z431	2Q4	Ro33	Wellcome	3D7	B
Z434	R2	K1	Mad 20	3D7	K, F
Z435	23	K1	Wellcome	3D7	F
Z439	J3	Mad 20	Mad 20	3D7	D
Z440	D4	Ro33, K1	Wellcome	FC27	G
Z442	J4	Mad 20	Mad 20	3D7	D
Z456	P5	Ro33	Wellcome	3D7	B
Z457	H16	Ro33, Mad 20	Wellcome, Mad 20	FC 27	E
Z458	2J6	Ro33, K1	Wellcome, Mad 20	FC27	F
Z740	V7	N.D.	N.D.	N.D.	N.D.

* N.D., Not done.

incubated with 250 μl of ethidium bromide solution (40 μg/ml) for 30 min at room temperature and washed in RPMI. The culture was synchronized by gel flotation (Jensen, 1978), the late-stage parasites were collected in the supernatant and washed in PBS supplemented with 2% foetal calf serum (FCS) and re-suspended in 5 ml of PBS with 2% FCS (2% FCS). Aliquots (100 μl) of this suspension were mixed with 5 μl of test plasma and incubated for 30 min at room temperature. After washing, the parasites were re-suspended to the original volume in 2% FCS and incubated with 2 μl of goat anti-human IgG antibody (DAKO, Glostrup, Denmark) for 30 min. After additional washing, the culture was re-suspended as before and 2 μl of rabbit anti-goat-IgG antibody (DAKO) conjugated to fluorescein isothiocyanate (FITC) was added, followed by incubation for an additional 30 min. After a final wash the parasites were re-suspended in 300 μl of 2% FCS and analysed on an Epics XL-MCL flow-cytometer (Coulter, Miami, FL, USA). The flow-cytometry data were analysed by PC lysys software (Becton Dickinson). Samples were gated on parasitized erythrocytes using forward and side scatter, and a gate defining cells containing ethidium bromide. The amount of IgG bound on the erythrocyte surface was then measured as the mean FITC fluorescence signal. For each cultured parasite isolate all plasma samples from Daraweesh were tested at the same time together with 5 samples from adult, malaria-exposed Gambian donors as positive controls. Individual plasma from 5 Danes and a pool of normal human serum from Danish blood bank donors served as negative controls. The negative control pool always gave a higher signal than any of the individual Danish plasma samples. When comparing the reactivity of different plasma samples to 1 parasite isolate (as in Figs 3 and 4) the reactivity of plasma from Daraweesh or Gambia is presented as the mean fluorescence signal obtained with the plasma minus the mean signal obtained with the pool of normal serum. When comparing results obtained with different isolates (as in Figs 5–7), the mean fluorescence of the 5 Gambian samples was calculated and the values of the test plasma presented as percentages of this value. Plasma reactivity was considered above cut-off if the reactivity was 1·25 times higher than in the Danish pool.

Statistics

Differences in proportions were analysed by Fisher's exact test. Parameter association was analysed by the Spearman rank order test. Differences between groups were analysed by *t*-test, when assumption of normal distribution was not violated, and otherwise by the Mann-Whitney rank sum test. SigmaStat software (Jandel Scientific, San Rafael, CA, USA) was used for the analysis.

RESULTS

Parasites

The genotypes of the parasites detected in the 16 donors who had a clinical malaria attack are shown in Table 1. In accordance with several other studies of parasite genetic diversity carried out in this area (Babiker *et al.* 1991*a*, *b*; Roper *et al.* 1998), the parasites were genetically diverse. The 2 samples that could not be typed, Z361 and Z740 had very low parasitaemias at the time of sampling. Based on the results of the genotyping and the ability to grow *in vitro*, 4 isolates (Z416, Z439, Z442, and Z457) were

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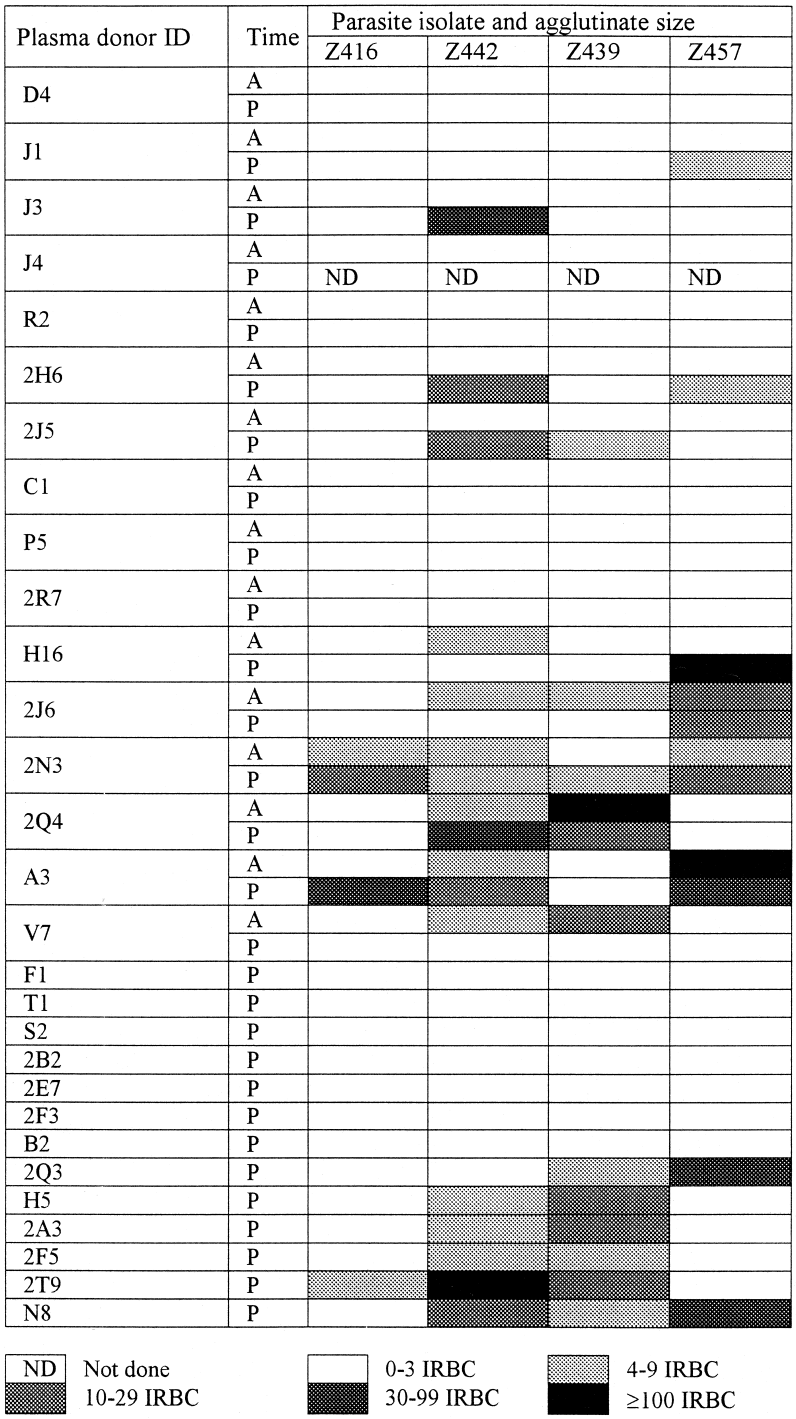

Fig. 1. Agglutination of parasite isolates Z416, Z439, Z442, and Z457 by plasma obtained from patients at diagnosis of malaria (A) and after the malaria season (P). Intensity of shading indicates the number of infected red blood cells (IRBC) agglutinated by the plasma in the largest agglutinates formed, as indicated in the bottom of the figure.

selected for further studying. The isolates Z439 and Z442 were chosen because the allelic typing indicated that they were monoclonal and genotypically identical, and different from the genotypes of the other 2 mutually distinct isolates, Z416 and Z457. Z439 and Z442 were collected from siblings living in the same hut, who were diagnosed as being clinically ill with malaria on the same day. Initially they were treated with chloroquine, but due to resistance they were both switched to Fansidar. There is therefore reason to believe that these 2 isolates are indeed genetically identical parasites originating from the bite of a single mosquito.

Agglutination

The 4 isolates were tested in agglutination assays using plasmas collected during acute episodes of

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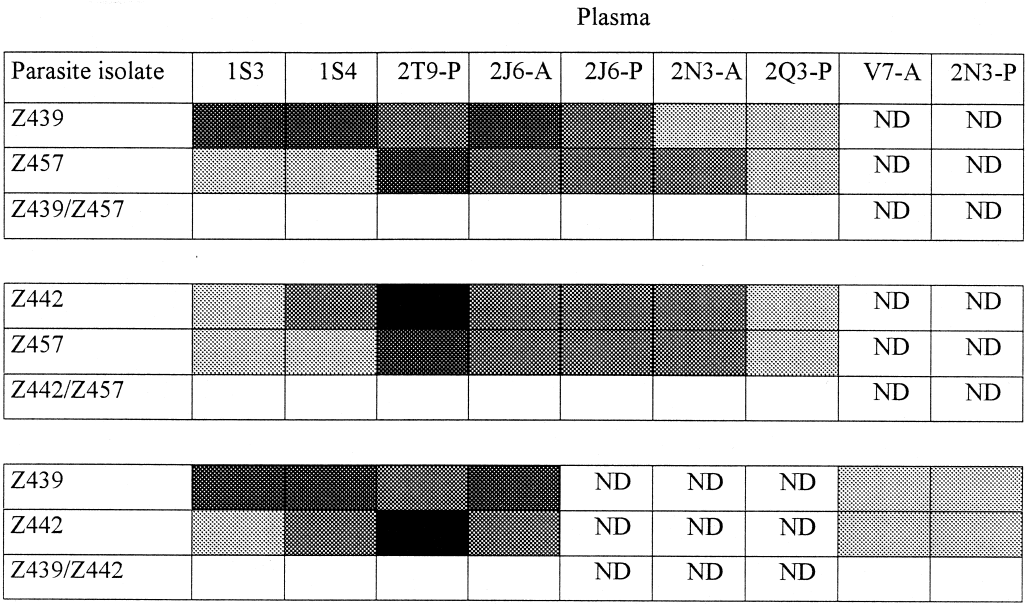

Fig. 2. Result of mixed agglutination assays. Each parasite isolate in the 3 sets of parasites tested (Z439/Z457, Z442/Z457 and Z439/Z442) was stained by 2 different fluorescent dyes and mixed. The parasites were incubated with plasma from 2 Gambians (1S3, 1S4) or plasma from individuals from Daraweesh obtained at diagnosis of malaria (A) or at the end of the malaria season (P). It is indicated whether agglutinates consisted of cells stained by the same colour (Z439, Z457, Z442, Z457, Z439, Z442) or cells stained with different colours (Z439/Z457, Z442/Z457, Z439/Z442). The intensity of shading reflects the size of the agglutinates, as indicated in the legend to Fig. 1.

malaria and at the end of the season from 16 individuals (Fig. 1). Post-season samples from 13 individuals who did not suffer from malaria during the season were also tested. Recognition of Z439 and Z442 correlated, since 65 % (11 of 17) of the samples recognizing Z442 also recognized Z439 and only 4 % (1 of 27) recognized Z439 but not Z442 ($P < 0{\cdot}001$, Fisher test). Seven of 17 samples (41 %) recognizing Z442 also agglutinated Z457, whereas only 4 of 27 samples (15 %) unable to agglutinate Z442 recognized Z457 ($P = 0{\cdot}075$, Fisher test). There was no association between recognition of Z439 and Z457 ($P = 0{\cdot}47$, Fisher test).

Mixed agglutination

Several of the plasma samples agglutinated more than 1 parasite isolate (Fig. 1). To test whether this was because the isolates expressed the same PfEMP1 serotype or because antibodies against different serotypes of PfEMP1 were present in the plasma, we tested several combinations of plasma and parasites in mixed agglutination assays (Fig. 2). The isolate combinations used were Z439/Z457, Z442/Z457 and Z439/Z442. The plasmas were from 2 adult Gambians and 7 individuals from Daraweesh selected because their plasma reacted to more than 1 parasite in the agglutination assay. Neither the genetically dissimilar (Z439/Z457 and Z442/Z457) nor the apparently genetically identical (Z439/Z442) pairs of parasites formed mixed agglutinates, indicating that the parasite isolates expressed different types of PfEMP1 and that antibodies against several serotypes of PfEMP1 were present in the plasmas.

Antibodies to autologous parasite isolates measured by agglutination assay and flow cytometry

The agglutination assay is difficult to perform on large panels of plasma and it does not allow a precise quantitation of the antibodies binding to the infected erythrocytes. We have developed a flow cytometric assay that measures antibodies bound to the surface of schizont-infected erythrocytes to overcome this difficulty (Staalsoe *et al.* 1999). Fig. 3 shows a comparison between results obtained by the 2 assays, testing plasma from the parasite donors collected at diagnosis of malaria and 2 months after treatment. In donor J3, the flow cytometry assay detected an increase of antibody levels to the autologous isolate Z439 and to lesser extent to isolate Z416, but not to isolate Z457. The agglutination assay did not detect these increases. We also detected acquisition of antibodies to the parasite isolate Z442 by donor J3, which could be detected by both flow cytometry and agglutination. A similar picture was seen in donor H16. With respect to 2J5, we could detect a response to the autologous parasite isolate Z416 by flow cytometry but not by agglutination. Isolate Z416 was only agglutinated by a few plasma samples (Fig. 1) but readily formed spontaneous rosettes (data not shown). We therefore compared the performance of

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Plasma donor	Time	Parasite isolate							
		Z416		Z439		Z442		Z457	
		FACS	Agg.	FACS	Agg.	FACS	Agg.	FACS	Agg.
J3	A	2		0		0		8	
	P	17		24		42		0	
J4	A	0		0		0		0	
	P	ND	ND	ND	ND	ND	ND	ND	ND
H16	A	41		38		32		15	
	P	49		16		41		100	
2J5	A	29		0		0		8	
	P	49		9		4		12	
Gambian adult		140	ND	175	ND	172	ND	72	ND

Fig. 3. Antibody to surface-exposed variable antigen on infected erythrocytes expressed by parasite isolates Z416, Z439, Z442, and Z457 in autologous plasma obtained at diagnosis of malaria (A) and 2 months after treatment (P). Plasma from an adult Gambian was used as positive control. The antibodies were measured by flow cytometry (FACS) and agglutination assay (Agg.). FACS values are mean fluorescence intensity in plasma minus the mean intensity in a pool of Danish serum as described in the Materials and Methods section. The intensity of shading reflects the size of the agglutinates, as indicated in the legend to Fig. 1.

Plasma donors	Agglutination	Agglutination in sodium citrate	Flow cytometry
NHS			00
J1-P			55
2J6-A			58
N8-P			62
2T9-P			65
C1-P			66
2F5-P			74
B2-P			82
2J6-P			83
A3-P			93
2N3-P			105
2N3-A			120

Fig. 4. Plasma agglutination of parasite isolate Z416 in the absence or presence of sodium citrate, and plasma surface reactivity to infected erythrocytes measured by flow cytometry. The plasma from individuals living in Daraweesh were obtained at diagnosis of malaria (A) and at the end of the malaria season in January (P). The flow cytometry values are calculated as described in the legend to Fig. 3. The intensity of shading indicates the size of the agglutinates (see legend to Fig. 1).

the isolate Z416 in flow cytometry and in agglutination assays with or without sodium citrate, which disrupts rosetting (Carlson *et al.* 1990). Fig. 4 shows that the presence of sodium citrate in the agglutination assays markedly increased the ability of the plasma to induce agglutination ($P = 0{\cdot}011$, paired *t*-test).

Typing of parasites using flow cytometry and plasma collected at the end of the malaria season

The results of the mixed agglutination assays indicated that the isolates Z439, Z442 and Z457 did not express the same PfEMP1 serotype at the time of assay. We therefore tested whether the ability of post-malaria season plasma to recognize the different isolates by flow cytometry correlated (Fig. 5). Considering all plasmas and all parasite combinations there was an association between the ability to recognize pairs of parasite isolates ($r = 0{\cdot}54$–$0{\cdot}91$, $P < 0{\cdot}007$ for all combinations of parasites, Spearman rank order test). The association was strongest when comparing parasite isolates Z439 and Z442, and when double-negative plasmas were excluded from the analysis, this was the only correlation which remained statistically significant ($r = 0{\cdot}81$, $P < 0{\cdot}007$). Plasma from some individuals reacted strongly with some isolates but not with others. This effect was most pronounced when comparing reactivity of Z457 to the other isolates. On the other hand, plasma for many donors recognized more than 1 parasite isolate at the end of the season, and since the mixed agglutination assays showed that the isolates did not have the same serotype, this indicates that the plasma contained antibodies recognizing several serotypes.

Acquisition of antibodies to antigens expressed on the surface of infected erythrocytes

The presence of antibodies to several serotypes could be the result of sequential acquisition of antibodies to individual serotypes by repeated exposure to new parasites and/or by simultaneous acquisition of antibodies to individual serotypes by repeated exposure to new parasites and/or by simultaneous acquisition of antibodies to several serotypes during the course of a single infection. To test whether a single infection resulted in production of antibodies against several serotypes we compared reactivities of plasma collected at the diagnosis of malaria and again after the malaria season 3–4 months later (Fig. 6). These patients were continually monitored both clinically and parasitologically, and given the very short eastern Sudanese malaria transmission season it is unlikely new blood infections were established after treatment of their initial infection. Many donors acquired the ability to react to more than 1 parasite indicating that antibodies to several serotypes were acquired during the course of a single infection. Furthermore, there

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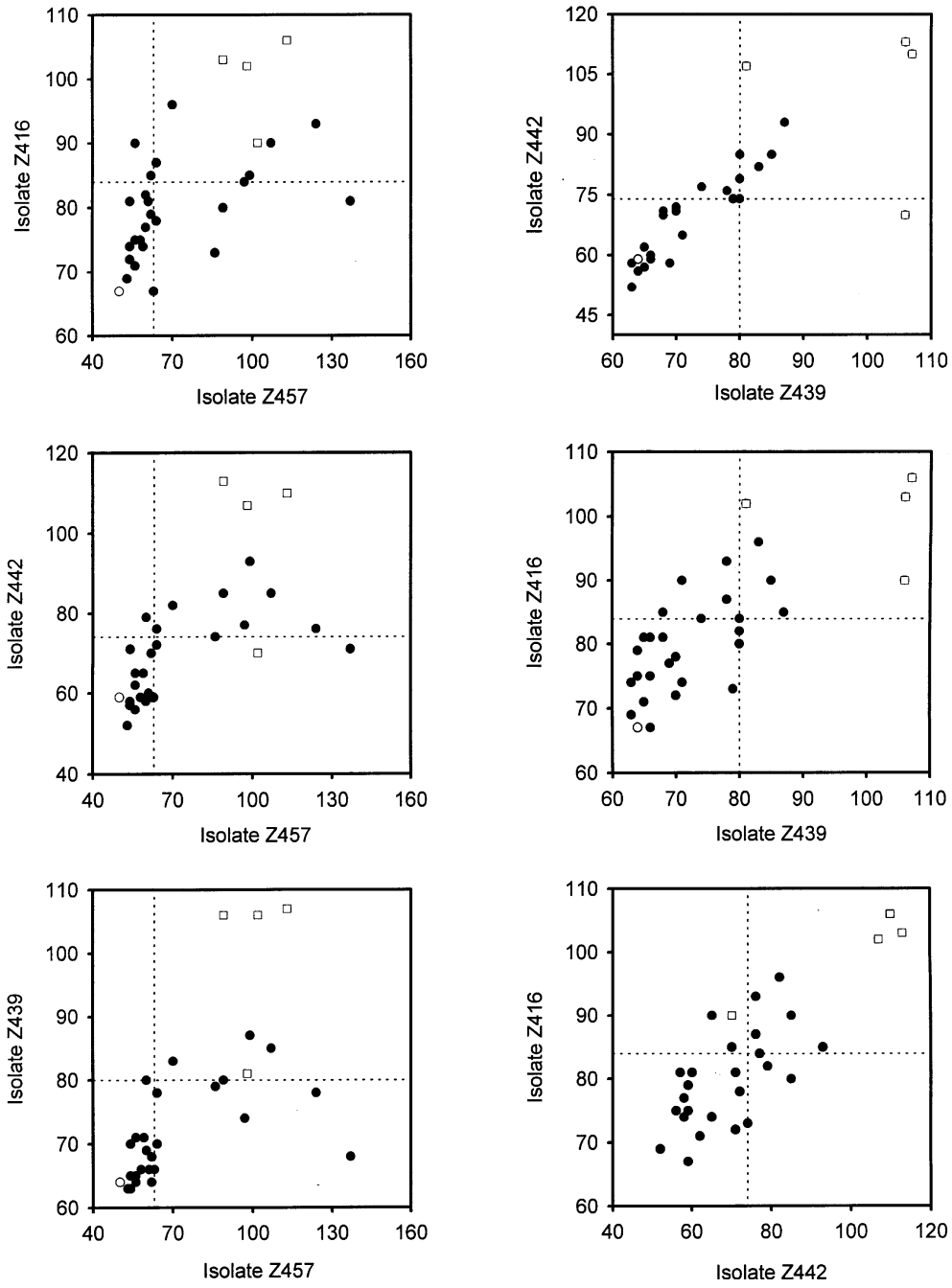

Fig. 5. Reactions to surface-exposed variable antigens on infected erythrocytes in plasma from Daraweesh (●) and in a pool of Danish normal serum (○). The plasma samples were collected at the end of the malaria season. Plasma samples from 4 Gambian adults are included as positive controls (□). Each panel shows the reactivity to 2 isolates of parasites in the same plasma. The reactivity of each plasma was calculated as a percentage of the mean fluorescence of the 4 Gambian donors. The dashed lines indicate cut-off levels defined as the reactivity in the pool of Danish serum $\times 1{\cdot}25$.

was a strong correlation between levels of acquired antibody to the *var* types expressed by Z439 and Z442 ($r = 0{\cdot}65$, $P = 0{\cdot}0069$, Spearman rank order test). The levels were not correlated when comparing Z439 versus Z416, but in this plot many double positive samples were found, indicating that antibodies to both isolates were acquired simultaneously although to different degrees. When comparing Z439 to Z457 the tendency was that antibodies were either acquired to neither of the isolates or to only 1 of them.

Antibodies to surface expressed antigens at the end of the malaria season

In order to estimate the amount of antibodies to the 4 different strains in an individual, the fluorescence values obtained from each post-season plasma sample when reacted with each of the 4 parasite isolates were added and divided by 4 (Fig. 7). The mean values were not associated with age ($P = 0{\cdot}37$, Spearman rank order test). When comparing the fluorescence intensity between individuals who

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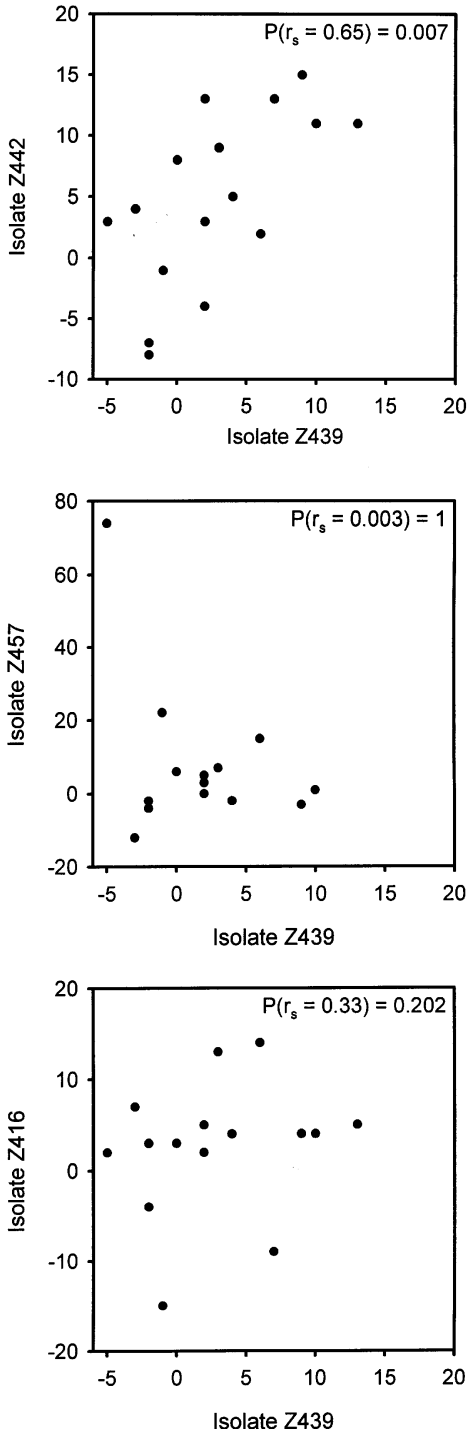

Fig. 6. Acquisition of antibodies to surface-exposed variable antigens on 3 pairs of parasite isolates by individuals in Daraweesh. The plasma was collected from individuals with malaria and from the same donors at the end of the malaria season. The result is the mean fluorescence in the end-of-the-malaria-season sample minus mean fluorescence in the acute sample. In order to be able to compare reactivity between isolates the values are given as a percentage of the reactivity in a pool of positive control plasma (see legend to Fig. 5 and Materials and Methods section).

suffered an attack of malaria during the season to those who did not, no difference could be detected ($P = 0{\cdot}22$, Mann–Whitney Rank Sum Test).

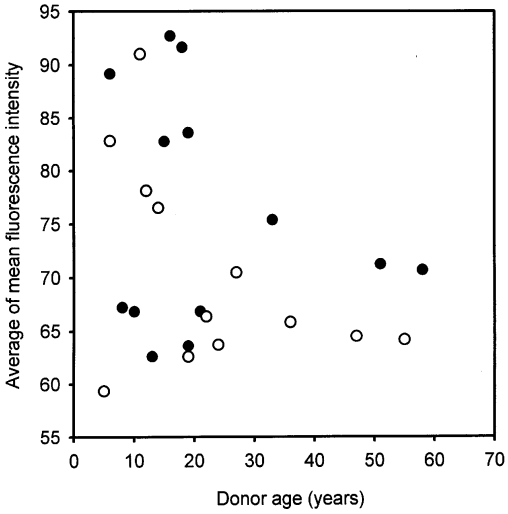

Fig. 7. Antibodies to variable surface-exposed antigens on infected erythrocytes in plasma collected at the end of the malaria season from donors who did (●) or did not have (○) malaria during the season. The values are the mean fluorescence intensity obtained in 4 isolates of parasites divided by 4. The reactivity to each parasite in plasma was calculated as a percentage of the reactivity in a pool of positive control plasma.

DISCUSSION

In Daraweesh the malaria season is short and the transmission intensity is low and unstable (Arnot, 1998; Theander, 1998). We have previously shown that many individuals from the village acquired antibodies agglutinating several genetically diverse parasite isolates during the malaria season (Elhassan *et al.* 1995; Giha *et al.* 1998). Assuming that the target for agglutinating antibodies are PfEMP1 (Baruch *et al.* 1995), this implies that challenge with 1 inoculum of parasites can induce antibodies recognizing PfEMP1 expressed on erythrocytes infected by different isolates of parasites. The possible explanations for this finding are: (a) that at least some of the parasites circulating in the village expressed the same serological variant of PfEMP1; (b) that antibodies in the plasma recognized conserved epitopes of PfEMP1 (Marsh & Howard, 1986; Staalsø *et al.* 1998) or (c) that the plasma contained antibodies of different specificity reacting with different variant epitopes of PfEMP1 (Newbold *et al.* 1992). To test these possibilities, we cultured parasites from 4 of the donors in the study. Two parasite isolates (Z439 and Z442) appeared genotypically identical and had the same drug-resistance phenotype. The 2 other isolates (Z416 and Z457) were genotypically different from Z439/Z442 and from each other. Using the mixed agglutination assay, the 3 parasite isolates tested in combination (Z439/Z442, Z439/Z457, Z442/Z457) expressed different agglutinating antigens, and the plasmas agglutinating more than 1 parasite contained antibodies recognizing several variants of the agglutinating antigens. This is in accordance with the finding

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of Newbold *et al*. (1992), who reported that serum from Gambian donors seldom form mixed agglutinates.

Despite the fact that Z439 and Z442 expressed different agglutinating antigens at the time of assay, there was a correlation between the ability of individual plasmas to agglutinate these 2 isolates. In order to quantify the reactivity to *P. falciparum* antigens expressed on the surface of infected erythrocytes, we developed an assay that measures the binding of antibodies to variant surface antigens on erythrocytes infected with late-stage parasites by flow cytometry (Staalsoe *et al*. 1999). This assay has several advantages over agglutination assays, an important one being that antibody reactivity to parasite strains which form rosettes that can prevent agglutination, can be measured.

When comparing the reactivity of individual plasmas collected at the end of the malaria season with the different combinations of isolates, different patterns emerged. There was a strong correlation between the ability to react to Z439 and Z442, and between Z439 and Z442 on the one hand side and Z416 on the other. In contrast, the presence of antibodies against Z457 did not predict the presence of antibodies against the other strains tested. By comparing antibody levels in samples obtained at diagnosis of malaria and at the end of the season, we found that individuals were more likely to simultaneously acquire antibodies to the 2 different serotypes expressed by Z439 and Z442 than to the serotypes expressed by Z439 and Z457 during infection. Antibodies to the serotypes expressed by Z439 and Z442 were also induced during infection with parasites carrying other genotypes than Z439/Z442. It therefore seems that, independently of genotype, parasites expressing the Z439 serotype were more likely to express the Z442 serotype during infection than the Z457 serotype.

This study confirms previous observations that agglutinating antibodies to several types of variant molecules on the surface of *P. falciparum*-infected erythrocytes are induced during both clinical and subclinical *P. falciparum* infections (Giha *et al*. 1998). Furthermore it demonstrates that infection with a genotypically distinct parasite isolate induces production of agglutinating antibodies capable of recognizing at least some of the serotypes expressed by unrelated parasite isolates. The parasite isolates studied here were a subset of more than 100 clinical infections present in the village in 1995 and our data therefore suggest the local repertoire of PfEMP1 is limited. We have previously shown that some blood-stage infections in Daraweesh run a subclinical course (Elhassan *et al*. 1995), but that it is impossible to predict from one year to the next who will be susceptible and who will be protected (our unpublished observations). It is conceivable that protection and susceptibility depends on the extent of overlap in the PfEMP1 repertoire between parasites in new inoculations compared to previous infections. Finally, our data support a model in which at least some common 'basic types' of PfEMP1 exist and are expressed during *P. falciparum* infections. Such 'basic types' appear to be immunogenic and to stimulate antibody responses, although these may be of short duration, particularly in the absence of stimulation by continuous infection (Giha *et al*. 1998; Cavanagh *et al*. 1998). Such antibody responses may then give rise to the cross-agglutinating responses observed here. This type of model to explain the sero-epidemiology of agglutinating antibody responses to parasitized erythrocytes is consistent with the most recent data on PfEMP1/*var* gene switching and cytoadherence. Using receptor panning assays evidence for a model in which one particular *var* gene variant is responsible for a particular cytoadherence phenotype has been obtained (Scherf *et al*. 1998). Such a 'one *var* gene type, one adhesion receptor' model combined with the observed ubiquity of such adhesive phenotypes of parasitized erythrocytes as CD36 binding (Ockenhouse *et al*. 1991; Newbold *et al*. 1997) implies that, for example, expression of a CD36-binding parasitized erythrocyte surface antigen is a common property of wild *P. falciparum* isolates, which may explain some of the cross-agglutination reactions we observed. This 'common PfEMP1 types' model is thus distinct from those in which PfEMP1 is proposed to confer enhanced and long-lasting protective immunity relative to sporozoite and merozoite antigens and thus to structure the parasites into strains that have little overlap between their PfEMP1 repertoires (Gupta & Day, 1994).

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