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A Longitudinal Study of Type-Specific Antibody Responses to *Plasmodium falciparum* Merozoite Surface Protein-1 in an Area of Unstable Malaria in Sudan[1,2]

David R. Cavanagh,[3]* Ibrahim M. Elhassan,† Cally Roper,* V. Jane Robinson,* Haider Giha,‡ Anthony A. Holder,§ Lars Hviid,¶ Thor G. Theander,¶ David E. Arnot,* and Jana S. McBride*

Merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* is a malaria vaccine candidate Ag. Immunity to MSP-1 has been implicated in protection against infection in animal models. However, MSP-1 is a polymorphic protein and its immune recognition by humans following infection is not well understood. We have compared the immunogenicity of conserved and polymorphic regions of MSP-1, the specificity of Ab responses to a polymorphic region of the Ag, and the duration of these responses in Sudanese villagers intermittently exposed to *P. falciparum* infections. Recombinant Ags representing the conserved N terminus (Block 1), the conserved C terminus, and the three main types of the major polymorphic region (Block 2) of MSP-1 were used to determine the specificity and longitudinal patterns of IgG Ab responses to MSP-1 in individuals. Abs from 52 donors were assessed before, during, and after malaria transmission seasons for 4 yr. Ags from the Block 1 region were rarely recognized by any donor. Responses to the C-terminal Ag occurred in the majority of acutely infected individuals and thus were a reliable indicator of recent clinical infection. Ags from the polymorphic Block 2 region of MSP-1 were recognized by many, although not all individuals after clinical malaria infections. Responses to Block 2 were type specific and correlated with PCR typing of parasites present at the time of infection. Responses to all of these Ags declined within a few months of drug treatment and parasite clearance, indicating that naturally induced human Ab responses to MSP-1 are short lived. ***The Journal of Immunology*****, 1998, 161: 347–359.**

Plasmodium falciparum malaria is a major tropical infectious disease, responsible for over 1 million deaths and around 500 million clinical cases per year worldwide. Several proteins specific for different stages of the parasite's life cycle are being studied for their potential as immunogens and vaccine targets (1). Merozoite surface protein-1 (MSP-1),[4] a polymorphic protein of approximately 190 kDa, is the major surface Ag of the invasive merozoite stage and thus an obvious vaccine candidate. Posttranslational proteolytic processing of MSP-1 generates fragments of 83, 42, 38, and 28–30 kDa, which persist as a noncovalently linked complex on the surface of mature extracellular merozoites (2–4) (Fig. 1). Secondary processing of the 42-kDa fragment produces a C-terminal 19-kDa fragment (MSP-1_{19}), which is retained on the surface of merozoites throughout invasion of erythrocytes, all other fragments being shed before or at this event (5, 6).

Partially purified *P. falciparum*-derived MSP-1 has protected monkeys from artificially induced malaria infections (7–9). *Aotus* monkeys immunized with MSP-1 of the Palo Alto isolate were completely protected from the lethal effects of challenge with the same parasite isolate (7). By contrast, a lower degree of protection was observed when monkeys were immunized with MSP-1 purified from the K1 isolate, then challenged with a heterologous parasite isolate (8). However, further studies showed that immunization with parasite-derived MSP-1 of the K1 isolate could protect *Saimiri* monkeys from lethal challenge with the nonhomologous Palo Alto isolate of the parasite (9). One of several problems in interpreting these conflicting results is that it is not known which parts of this large protein are important in the protective immunity induced by the whole Ag. Immunizations of monkeys with recombinant MSP-1-derived protein fragments also induced protection from experimental challenge (9–13). Taken together, these studies have indicated that protective immunity can be induced with proteins derived from either the N- or the C-terminal regions of MSP-1. Such a view is supported by experiments showing that mAbs specific for either a variant epitope in the N-terminal Block 2 region, or conserved epitopes in the C-terminal MSP-1_{19} fragment, can inhibit parasite growth in vitro (5, 14). A recombinant MSP-1_{19} and a longer MSP-1_{42} are immunogenic in animals (15, 16) and elicit Abs that inhibit parasite growth in vitro (13).

*Institute of Cell, Animal and Population Biology, Division of Biological Sciences, University of Edinburgh, Edinburgh, Scotland, United Kingdom; †Institute of Endemic Diseases and ‡Department of Biochemistry, University of Khartoum, Sudan; ¶Centre for Medical Parasitology at Institute of Medical Microbiology and Immunology, University of Copenhagen, and Department of Infectious Diseases, Copenhagen University Hospital, Copenhagen, Denmark; and §National Institute for Medical Research, The Ridgeway, London, United Kingdom

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[2] The nucleotide sequences of the K1-like and MAD20-like proteins have the GenBank accession numbers AF034636, AF034792, and AF034635, respectively.

[3] Address correspondence and reprint requests to Dr. D. R. Cavanagh, Institute of Cell, Animal and Population Biology, Division of Biologic Sciences, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh, EH9 3JT, Scotland, U.K. E-mail address: cavanagh@srv0.bio.ed.ac.uk

[4] Abbreviations used in this paper: MSP-1, merozoite surface protein-1; GST, glutathione *S*-transferase.

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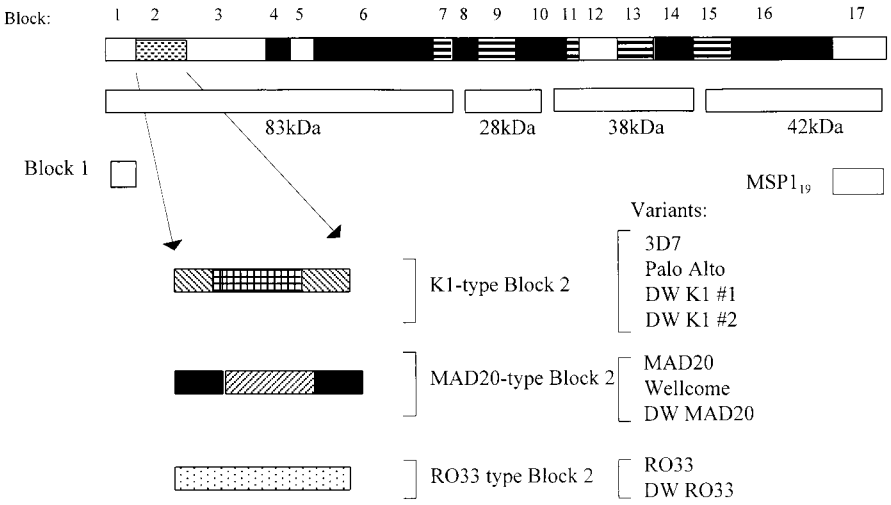


FIGURE 1. Schematic representation of MSP-1 of *P. falciparum* and of recombinant MSP-1 Ags (adapted from Ref. 41). The division into 17 blocks is as outlined by Tanabe et al. (17); blocks of conserved sequences are denoted by open boxes; regions of dimorphic or semiconserved sequences are denoted by full or hatched boxes, respectively; and the polymorphic Block 2 region is shown as a speckled box. Shown below the full-length protein is the scheme of natural processing of MSP-1. Recombinant Ags of Block 1 and Block 17 (on scale) and of Block 2 variants (enlarged) derived from MSP-1 alleles of the indicated *P. falciparum* isolates are shown below the main diagram.

Sequence comparisons of *MSP-1* alleles indicate that the *P. falciparum MSP-1* gene can be divided into 17 distinct Blocks that encode conserved, semiconserved, or variable regions of the protein (17, 18) (Fig. 1). With the exception of the highly polymorphic N-terminal Block 2 region, the nonconserved sequences are dimorphic and can be grouped into one or other of two major families represented by the MAD20 and the Wellcome prototypes (17). Allelic polymorphism in Block 2 is much more extensive, with over 50 different sequence variants identified. Nevertheless, these sequences all fall into three main types represented by variants originally described in the K1, MAD20, and RO33 isolates (18). Block 2 variants of the K1-like and MAD20-like types contain variable tri- or hexapeptide repeats (17, 19, 20), whereas Block 2 of the RO33 type is a nonrepetitive sequence that varies little between isolates (21).

Interest in naturally acquired human immune responses to MSP-1 has focused on the C-terminal region (reviewed in Ref. 22). Abs to this region are found in the majority of malaria-exposed individuals from endemic areas (23–25). Correlation between high levels of Ab to the C-terminal region and "protection" from clinical malaria symptoms in humans has been reported (26, 27), although protection against reinfection was not observed. Human Ab responses to other regions of MSP-1 are less well studied. Two regions from the N-terminal end of MSP-1, the semiconserved sequence Block 1 and the highly polymorphic Block 2, merit investigation. Block 1 contains the amino acid sequence YSLFQKEKMVL included in the Spf66 vaccine (28–30). Studies on human Ab responses to MSP-1 following natural infections suggest that both polymorphic and dimorphic sequences of the molecule may play a role in inducing immunity (31, 32). Increased levels of IgG against an N-terminal fragment of MSP-1 distinguished Gabonese patients who had cleared infections from those who had persistent infection (33). Other studies found that the lower the level and the shorter-lived the humoral response to N-terminal regions of MSP-1, the higher the risk of subsequent reinfection (32). Contradicting this finding, Tolle et al. reported that Abs to several regions of the MSP-1 molecule, including the polymorphic Block 2 region, were correlated with increased risk of reinfection and/or decreased ability to control parasitemia (34).

A possible significance of the allelic variation in Block 2 sequences for parasite "immune evasion" has been proposed on theoretical grounds (35). However, there is no direct evidence for immune responses specific for Block 2 variants, and neither the surveys of MSP-1 sequence diversity nor of seroepidemiology have considered whether the parasite types present in infections (usually assayed by PCR detection of genotypes) actually induce type-specific Ab responses in the same individuals. We have therefore investigated these problems in this study.

To analyze the specificity of human immune responses to different sequence forms of MSP-1, recombinant proteins representing the extreme N-terminal Block 1 and the three main types of the Block 2 region were produced. These Ags were used in ELISAs to measure MSP-1-specific Abs in villagers from an area of seasonal malaria transmission in eastern Sudan. Longitudinal samplings of a cohort of 52 villagers were performed over a 4-yr period to establish whether or not the Block 1 and Block 2 regions of MSP-1 are immunogenic during the course of a natural infection. The specificity and duration of Ab responses to the polymorphic and conserved regions of the MSP-1 molecule were determined. Longitudinal responses to these Ags were correlated with close clinical and parasitologic surveillance of each individual. This is the first study to correlate type-specific Ab responses with the presence of particular parasite MSP-1 types in malaria-infected individuals.

Materials and Methods

Study area

This longitudinal study was conducted in the village of Daraweesh, Gedaref State, eastern Sudan (population: ~400) (36). The major activity of the village is farming sorghum and sesame. The climate is Sahelian with a June to September rainy season (average 180 mm) and a long, dry season (September to June). Malaria in eastern Sudan is mesoendemic and unstable (37). The frequency of malaria cases peaks between October and November, with marked variations in the severity of annual outbreaks (38). *Plasmodium falciparum* is the major species of malaria parasite, accounting for 95% of all malaria cases. The inhabitants of Daraweesh have participated in a study of factors causing clinical malaria since 1988, including this present work on the development of immunity to defined regions of MSP-1.

Study cohort and sampling strategy

The study is based on 52 people (born between 1963–1987) who do not possess the sickling allele of the α hemoglobin gene and whose clinical histories and malaria infection experience have been followed since 1990. Malaria infections were detected by Giemsa staining and microscopic examination of blood samples donated during cross-sectional surveys of the village population at the beginning (September) and end (January) of the malaria transmission season each year. Blood samples were also taken from the individuals during and after episodes of illness with symptoms suggestive of malaria and/or a body temperature greater than 37.5°C. Malaria diagnosis was made by blood film examination. Those with *Plasmodium*-positive slides were classified as having a clinical episode of malaria. The patients were treated with chloroquine, followed by sulfadoxine/

pyrimethimine treatment in cases of apparent failure to respond to chloroquine. Individuals who had *Plasmodium*-negative blood films were considered "without malaria" that season. This monitoring of malaria cases was performed by the study's health team, including a doctor visiting the village every second day during the malaria season.

Anti-MSP-1 Abs were assayed in 487 plasma samples collected from the 52 donors over a 4-yr period (1991–1995). In addition to the sampling protocol described above, dry season samples were collected in June 1994 and June 1995 to estimate the persistence of Ab responses from the previous malaria transmission season. From September 1993 onward, PCR detection and MSP-1 typing of *P. falciparum* infections was performed on DNA extracted from RBCs taken from individuals at the collection points described above. All blood samples were obtained after informed consent, under the approval of the Sudanese Ministry of Health and the Ethics Committee of the Faculty of Medicine, University of Khartoum. Plasmas were stored at −20°C before use. Control sera of Europeans who had not been exposed to malaria infection were from healthy adult donors to the Scottish Blood Transfusion Service.

PCR amplification and typing of MSP-1 *gene fragments*

Genomic DNA from parasites present in infected individuals was purified for PCR as described (39) and used as template for Block 2 amplification. Three Block 2 type-specific amplification reactions (K1 type, MAD20 type, and RO33 type) were performed after an initial amplification of a larger fragment spanning Blocks 1 to 3 of the *MSP-1* gene, in a nested PCR system, essentially as described (57). Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 2 mM each dNTP, 2.5 U *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN) and 1 mM each of the following pairs of primers: forward primer (5′-CTGGATCCAATGAAGAAGAAATTACT-3′) and reverse primer (5′-GGGAATTCTTAGCTTGCATCAGCTGGAGG-3′) were used to amplify K1-like Block 2 regions; forward primer (5′-CTGGATCCAATGAAGGAACAAGTGGA-3′) and reverse primer (5′-GGGAATTCTTAACTTGAATTATCTGAAGG-3′) for Block 2 regions of MAD20-like types; and forward primer (5′-CTGGATCCAAGGATGGAGCAAATACT-3′) and reverse primer (5′-GGGAATTCTTAACTTGAATCATCTGAAGG-3′) for the RO33-like Block 2 regions. The underlined portions of each primer contain restriction endonuclease sites used in cloning Block 2 fragments into the expression vector pGEX 2-T. A PCR cycle of 95°C, 90 s; 50°C, 15 s; 72°C, 45 s was repeated for 35 to 40 cycles in each case. DNA fragments generated in each of the type-specific amplification reactions were resolved on 2% agarose gels.

Recombinant MSP-1 Ags

Four new Block 2 proteins were derived specifically from parasites present in Daraweesh villagers in October 1994. Two Daraweesh K1-like sequences, one MAD20-like sequence and one RO33-type sequence, were cloned and expressed in *Escherichia coli* as recombinant proteins fused to the C terminus of glutathione *S*-transferase (GST) of *Schistosoma japonicum* using the pGEX-2T vector (40), essentially as described (41). These proteins were designated DW K1 Block 2 no. 1, DW K1 Block 2 no. 2, and DW MAD20 Block 2, and DW RO33 Block 2. The nucleotide sequences of the K1-like and MAD20-like proteins have the GenBank accession numbers AF034636, AF034792, and AF034635, respectively. DW RO33 Block 2 was found to have an identical sequence to the published RO33 sequence (21). Block 1 (MAD20 and Palo Alto) and all other Block 2 (3D7, Palo Alto, MAD20, Wellcome, and RO33) recombinant proteins were described earlier (41). These proteins all induce animal Abs that recognize parasite-produced MSP1 with specificities as appropriate for distinct MSP-1 alleles expressed by a range of *P. falciparum* isolates (41).

A pGEX construct that encodes a GST fusion protein containing most of Block 17 and corresponding to the 19-kDa C-terminal fragment of MSP-1 (MSP-1_{19}) has been described earlier (42). The recombinant MSP-1_{19} (Asn^{1631} to Asn^{1726} of the Wellcome isolate) has a disulfide-dependent antigenic structure undistinguishable from that of *P. falciparum*-derived MSP-1_{19} (23, 42).

Enzyme-linked immunosorbent assay

Human sera were tested by ELISA for the presence of IgG Abs able to recognize the recombinant MSP-1 fragments. Wells of 96-well plates (Immulon 4; Dynatech, Chantilly, VA) were coated with 50 ng of recombinant Ags in 100 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.3) overnight at 4°C. The wells were washed three times in washing buffer (0.05% Tween-20 in PBS). Unoccupied protein binding sites were blocked with 200 μl per well of blocking buffer (1% (w/v) skimmed milk powder in washing buffer) for 5 h at room temperature and again washed three times. Human plasma diluted 1:500 in the blocking buffer (100 μl per well) was added to duplicate Ag-coated wells and incubated overnight at 4°C. After three washes, the wells were incubated for 3 h at room temperature with 100 μl per well of horseradish peroxidase-conjugated rabbit anti-human IgG (1:5000) (Dako, High Wycombe, U.K.). Plates were washed three times before incubating for 15 min at room temperature with 100 μl of substrate (0.1 mg ml^{-1} *o*-phenylenediamine; Sigma, St. Louis, MO; 0.012% H_2O_2) in development buffer (24.5 mM citric acid monohydrate and 52 mM Na_2HPO_4, pH 5.0). The reaction was stopped by the addition of 20 μl of 2 M H_2SO_4, and OD was measured at 492 nm. Corrected OD values for each plasma sample were calculated by subtracting the mean OD value of wells containing control GST protein alone from the mean OD value obtained with each test MSP-1 Ag.

Cut-off values at which binding of Ab from malaria-exposed individuals was regarded as significantly above background were calculated as the mean plus 3 SDs of OD readings obtained with sera from 37 Scottish blood donors with no history of exposure to malaria. Cut-off OD values for each Ag were as follows: MAD20 Block 1, 0.119; Palo Alto Block 1, 0.277; 3D7 Block 2, 0.428; Palo Alto Block 2, 0.188; DWK1 Block 2 no. 1, 0.163; DWK1 Block 2 no. 2, 0.133; MAD20 Block 2, 0.125; Wellcome Block 2, 0.180; DW MAD20 Block 2, 0.161; RO33 Block 2, 0.152; DW RO33 Block 2, 0.122; MSP-1_{19}, 0.133.

Competition ELISA was used to assess whether human anti-Block 2 Abs were specific for particular Block 2 variants or more generally cross-reacted with other Block 2 Ags within each type. Aliquots (100 μl) of selected sera diluted 1:500 were first reacted with 0 to 10 μg/ml of soluble competing Ag, i.e., with up to 20-fold excess over the 50 ng plate-bound Ag, then tested on the plate-bound Ag overnight. This was followed by washing and incubation with a horseradish peroxidase-conjugated second Ab, as described above.

Statistical methods

Differences between frequencies of Ab response were tested by χ^2 test, or by Fisher's exact probability test, where appropriate. Correlations between OD values for Ab reactivities with pairs of individual Ags were calculated as Spearman's rank correlation coefficients.

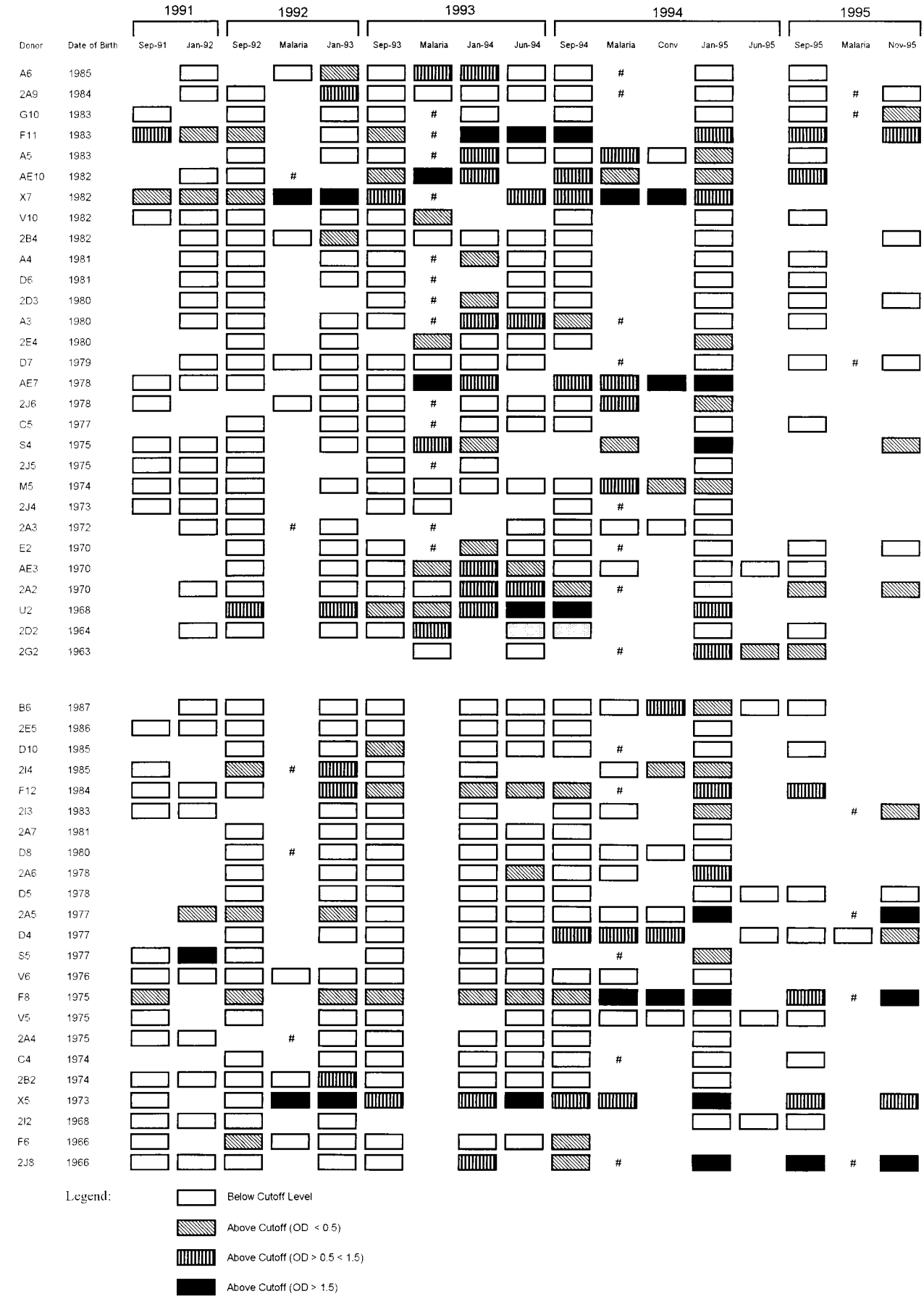
Results

Longitudinal survey of Ab recognition of MSP-1

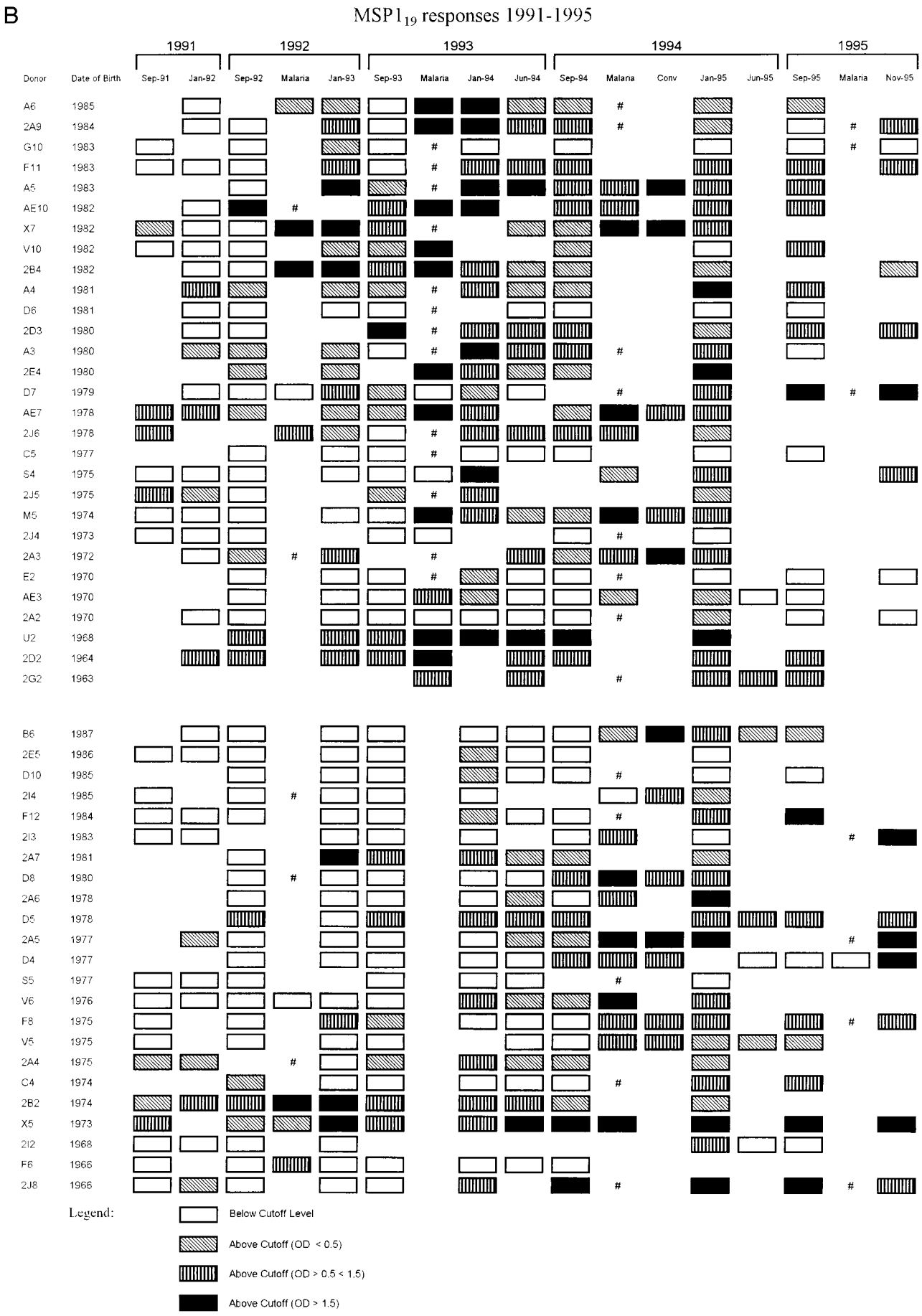
IgG Abs in plasmas collected from 52 individuals between 1991 and 1995 were detected by ELISA with recombinant MSP-1 Ags. Summaries of the individuals' clinical malaria experience during this period and of their Ab responses to any Block 2 Ag, and to the C-terminal fragment MSP-1_{19}, are shown in Figure 2, *A* and *B*, respectively. No malaria infections were seen in the cohort during a severe drought in 1990 and 1991 (Fig. 3). Between September 1991 and January 1992, detectable rises in Abs to either part of MSP-1 were seen in only three cohort members (S5, 2B2, and 2J8), although a surprisingly high proportion of the cohort (15 of 38, 34%) had low Ab levels despite the drought. Malaria morbidity increased in 1992 and reached its highest level in 1994 (since surveillance began in 1988), when rainfall in the area returned to normal levels (Fig. 3). This was reflected in the observed increases

FIGURE 2. Summary of detectable IgG specific for recombinant MSP-1 Ags in 52 individuals over 4 yr. Individuals and their dates of birth are listed in the first two columns. Each box indicates a single plasma sample, tested against all 9 variants of the Block 2 Ags (*summary A*) and against MSP-1_{19} (*B*). Ab reactivity of each sample as measured by ELISA is denoted by the pattern of shading. Empty boxes indicate no detectable Ab above European cut-off levels (mean OD plus 3 SDs, of either any Block 2 or C-terminal MSP-1_{19} Ag, calculated from sera from 37 European controls). Diagonal striped boxes indicate OD levels <0.5 above the cut-off levels; vertical striped boxes indicate OD 0.5 to 1.5 above cut-offs; black boxes indicate OD over 1.5 above cut-offs. In columns headed "Malaria," hash (#) marks indicate documented malaria episodes where no plasma sample was available for testing. The column headed "Conv" indicates plasma samples collected in convalescence 30 days after clinical malaria episodes.

A



B



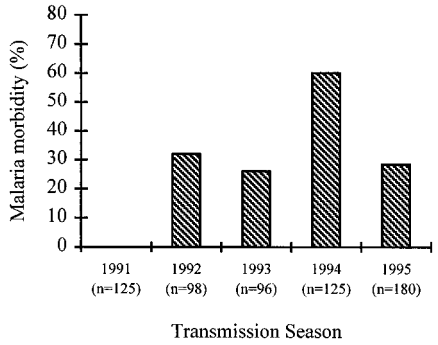


FIGURE 3. Incidence of malaria morbidity in Daraweesh, 1991 to 1995. The percentage of village members who had at least one clinical episode per malaria transmission season is shown for the 4 yr of survey. The number of people followed each year is shown in parentheses.

in the overall levels of Ab to both the polymorphic Block 2 Ags (Fig. 1*A*) and the C-terminal MSP-1_{19} (Fig. 2*B*) from January 1992 to 1995.

When the cohort is divided into two groups according to recorded malaria cases in each transmission season, namely individuals who had a malaria episode and those who had not, Ab responses to MSP-1 Ags differ significantly between these groups (Table I). A positive response to any particular Ag was defined as a rise of at least fourfold in detectable IgG after malaria infection when compared with each individual's preinfection levels. Rises in IgG to MSP-1 Ags were detected in 20 of 29 malaria cases in the 1993 transmission season, and in 20 of 33 individuals who had clinical malaria in the 1994 season. The frequency of rises in IgG levels to any of the MSP-1 Ags was much lower in individuals who did not have a clinical episode, and in whom pre- and postmalaria transmission season levels were compared. In 1993, only 3 of 22 of such individuals (V6, 2A4, and 2J8; Fig. 2) showed increased Ab levels against any MSP-1 Ag. Similar results were seen in 16 apparently healthy individuals during the 1994 season, with two rises in IgG specific for the C-terminal MSP-1_{19} (A4 and 2E4; Fig. 2). However, at least two of these Ab-positive individuals, V6 (1993) and 2E4 (1994), were infected asymptomatically since they had subpatent PCR-detectable parasites in posttransmission (January) blood samples. The differences in the incidence of Ab responses between the two groups were highly significant (Fisher exact probability tests; MSP-1_{19} 1993, $p = 0.00015$; 1994, $p = 0.00045$; Block 2 Ags 1993, $p = 0.00126$; 1994, $p = 0.00176$). Thus, in this cohort, under the described epidemiologic circumstances, when there was a detectable response to the Block 2 and/or MSP-1_{19} Ags, it almost always followed a clinical malaria episode, and was only rarely observed in individuals who had not suffered a clinical malaria attack. Ab responses to the conserved Block 1 Ags were rarely seen over the 4-yr study, with only five donors showing transitory levels of detectable Ab in postinfection samples (data not shown).

Frequency of Ab responses to polymorphic Block 2 Ags vs conserved C-terminal Ags

Following documented *P. falciparum* malaria episodes, the overall frequency of Ab responses to all Block 2 types combined was lower than that to the C-terminal MSP-1_{19} (Table I). When malaria transmission restarted after the 1992 rains, malaria episodes occurred in 15 cohort members that season. Eleven of these 15 individuals responded to the C-terminal MSP-1_{19}, and only 7 responded to any Block 2 Ags. In the 1993 transmission season, 29 individuals had at least one malaria attack. Increased IgG levels against MSP-1_{19} were observed in 20 of these individuals, whereas only 13 showed detectable increases in Ab reactivity to any Block 2 Ag over the same period. In the 1994 transmission season, 33 individuals had clinical malaria. Twenty responded by increased IgG specific for the C-terminal MSP-1_{19}, compared with 13 individuals who had increased levels of IgG specific for one or more Block 2 Ags.

In the 1992 season, of 7 individuals responding to Block 2, 2 responded specifically to the K1 type, 4 to the MAD20 type, and 1 to the RO33 type. No one responded to more than one Block 2 type. In the 1993 season, of the 13 individuals responding to Block 2, 6 showed specific reactivity against the K1 type, 5 against the MAD20 type, and 4 against the RO33 type (Table I). Thus, in this year, 3 individuals had increased Ab levels against more than one Block 2 type. In 1994, of 13 individuals, 5 showed reactivity against the K1 type of Block 2, 6 responded to the MAD20 type, and 6 to the RO33 type (Table I). Therefore, 5 individuals had Abs against more than one Block 2 type in 1994.

Patterns of response to MSP-1 in infected individuals

Longitudinal patterns of Ab responses were analyzed in 68 of the 77 malaria cases seen between 1992 and 1994 (since either pre- or postmalaria plasma samples were not available in 9 cases). Three different patterns of anti-MSP-1 response to *P. falciparum* infections were seen. The first pattern was observed in a minority of individuals (3 cases in 1993–1994 and 5 cases in 1994–1995) who had no detectable Ab response to MSP-1, despite documented clinical malaria episodes (Fig. 4*A* shows an example, individual E2). The second pattern is represented by individuals who responded well to the conserved C-terminal Ag MSP-1_{19}, but had no detectable Abs to the polymorphic Block 2 (23 of 68 cases during 1992–1994). Donor D8 (Fig. 4*B*) illustrates this type of response. The third and most common pattern consisted of responses to both Block 2 and the C-terminal Ags (37 of 68 documented malaria infections in 1992–1994). In the majority of these cases (32 of 37), the response to Block 2 was directed against a single type (e.g., donor F11, Fig. 4*C*). In a few cases (5 of 37), responses to more than one type of Block 2 Ag were observed. For example, donor F8 (Fig. 4*D*) responded to both the MAD20 and K1 Block 2 types in 1994, with reactivation of the anti-K1 response following a second infection in 1995. Taking the data as a whole, after clinical malaria infections, plasma Abs recognizing the conserved C-terminal Ag MSP-1_{19} were always more common than Abs against any Block 2 region (Table I and Fig. 2).

Correlation between Block 2 genotypes of infecting parasites and Block 2-specific Ab responses

From September 1993, the *P. falciparum* parasites present in 44 infections were genotyped by PCR for *MSP-1* Block 2, although anti-Block 2 responses were observed in only 29 of these infections. Comparison between the Block 2 specificity of the hosts' Ab responses and Block 2 type of infecting parasites is further complicated by the fact that some patients had infections in which single parasite types were detected, whereas others were clearly infected by more than one parasite clone. Of 18 PCR-typed malaria cases in the 1993 transmission season, 4 were multiple clone infections and 14 single clone infections as detected by PCR genotyping of *MSP-1* Block 2. In 1994, 13 multiple clone infections and 13 single clone infections were found.

To analyze the relationship between Ab response and PCR genotypes, concordant Ab responses were defined as those where the

Table I. *Incidence of specific Ab responses to different regions of MSP-1 in 52 individuals over three malaria transmission seasons*

Number responding to	Clinical Malaria			No Clinical Malaria		
	1992/3	1993/4	1994/5	1992/3	1993/4	1994/5
C-Terminal MSP-1_{19}	11/15 (73%)	20/29 (69%)	20/33 (61%)	4/33 (12%)	3/22 (14%)	2/18 (11%)
Block 2 antigens	7/15 (47%)	13/29 (45%)	13/33 (39%)	2/33 (6%)	1/22 (4.5%)	0/18 (0%)
K1-type	2/15 (13%)	6/29 (21%)	5/33 (15%)	0/33 (0%)	1/22 (4.5%)	0/18 (0%)
MAD20-type	4/15 (27%)	5/29 (17%)	6/33 (18%)	1/33 (3%)	1/22 (4.5%)	0/18 (0%)
RO33-type	1/15 (7%)	4/29 (14%)	6/33 (18%)	1/33 (3%)	0/22 (0%)	0/18 (0%)

[a] The cohort is divided into individuals with documented clinical malaria and those with no documented malaria in the 1992 to 1994 seasons. The number of individuals as a fraction of each group total (percentages in parentheses) showing a greater than fourfold increase in detectable IgG specific for the C-terminal conserved antigen MSP-1_{19} are shown in the upper row. The second row shows the number and percentage of individuals in whom a detectable IgG increase was seen against any Block 2 antigen, with individuals specifically recognizing each type of Block 2 shown in the lower three rows.

specificity of detectable Block 2 Abs matched at least one of the Block 2 types detected by PCR analysis. Discordant responses were defined as those where the Ab response to a Block 2 type did not match the detected PCR genotype. The specificity of anti-Block 2 Ab matched the Block 2 type of parasites detected in the same blood sample in 24 of 29 Ab-positive cases (9 in 1993–1994 and 15 in 1994–1995). PCR-typed infections of donors who had Block 2 Ab responses of a discordant type accounted for 17% of cases (3 in 1993–1994 and 2 in 1994–1995). There was thus a significant correlation between parasite Block 2 type and specificity of the subsequent anti-Block 2 response (results from the two transmission seasons combined, $\chi^2 = 12.45$, $p < 0.001$). This indicates that the type-specific Ab responses were induced by the infecting parasites usually detected at the time of the malaria episode.

Examples of anti-Block 2 Ab profiles in individuals with PCR-typed parasites are shown in Figure 5. Figure 5*A* shows a response of donor D4 to K1-type Block 2 following a mixed infection with K1- and RO33-type parasites in the 1994 season. K1-type parasites were detected in three samples taken before and during the acute malaria episode (October). Ab against K1-type Block 2 was first detected in September, before clinical symptoms appeared, persisted until November, but was undetectable by the following June. There was no response to RO33 Block 2 of the other parasite associated with this clinical episode. A second infection in October 1995 (again K1 and RO33 Block 2 types), was followed by a rise in Ab to the conserved C-terminal MSP-1_{19} but there was no detectable response to any Block 2 Ag. Figure 5*B* shows the response of donor A3, who was infected with parasites of MAD20 Block 2 type in the 1993 season and produced specific anti-MAD20 Abs, i.e., a response concordant with the infecting parasite type. In this donor, asymptomatic persistence of parasites of the MAD20 type was detected by PCR in both April and June 1994, and MAD20 Block 2-specific Abs then also persisted until September 1994.

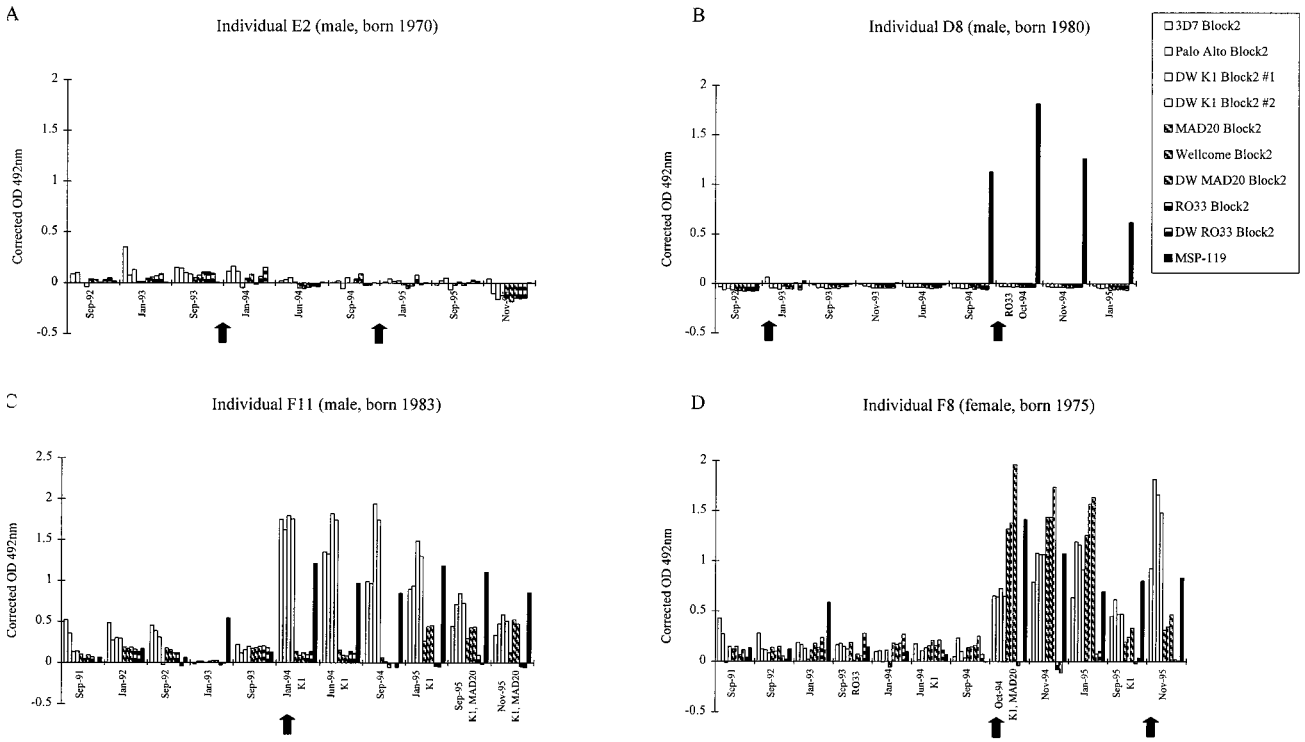


FIGURE 4. Patterns of Ab response to MSP-1 in cohort members, 1991 to 1995. Three typical patterns of detectable IgG response to Block 2 and/or MSP-1_{19} in *P. falciparum* infections are illustrated by examples. *A,* Anti-MSP-1 profile in an individual with no Ab response to documented malaria episodes. *B,* Response to MSP-1_{19} with no detectable anti-Block 2. *C,* Response to MSP-1_{19} and a Block 2 type in an infection with parasites of a single Block 2 type. *D,* Response to MSP-1_{19} and two different Block 2 types in a mixed infection with parasites of different Block 2 types. Arrows on the *x*-axis indicate clinical malaria infections in the individuals.

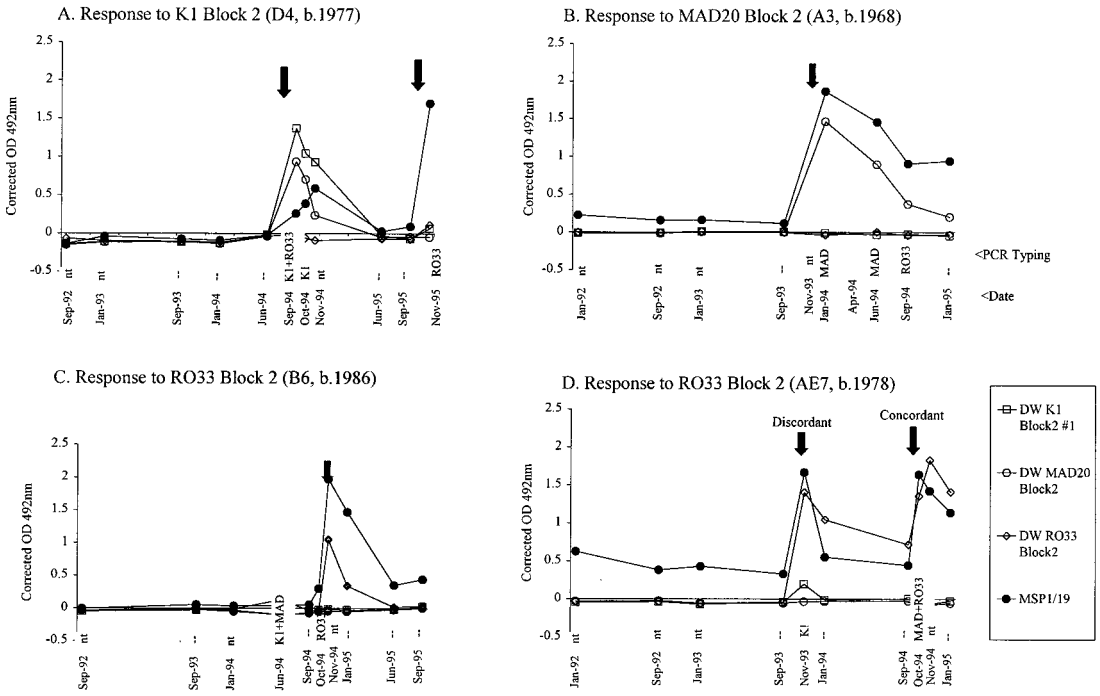


FIGURE 5. Parasite genotypes of MSP-1 Block 2 vs specificity of Ab responses to Block 2. Ab response profiles to Block 2 and MSP-1_{19} are shown for four individuals. Sample dates and PCR Block 2 genotypes of infecting parasites are shown along the *x*-axis, with Ab reactivity expressed as OD. Vertical arrows indicate clinical malaria episodes. Open symbols represent Ab levels to the three main Block 2 types. Closed circles represent Ab levels against MSP-1_{19}. *A*, Concordant Ab response to K1-like parasites. *B*, Concordant Ab response to MAD20-like parasites. *C*, Concordant Ab response to RO33-like parasites. *D*, A discordant RO33-specific Ab in the presence of K1-like parasites one year, followed by a concordant Ab response to RO33 parasites the following year.

Figure 5*C* shows the anti-Block 2 response of donor B6, who produced specific anti-RO33 Abs after a PCR-genotyped RO33 infection. The anti-RO33 Block 2 Abs fell shortly after the malaria episode, when drug treatment led to rapid parasite clearance following infection, as blood samples over the following month tested PCR negative. *D* illustrates two anti-Block 2 responses of donor AE7. A discordant response to the RO33 Block 2 type was seen after an infection with K1 type parasites in October 1993, and a concordant boost of the same specificity to a mixed infection, including RO33-type parasites, occurred 1 yr later. The rise in specific Ab to RO33 Block 2 in 1993 may have been a response to a minority parasite population that was not detected at the time by PCR genotyping. Alternatively, there may have been an asymptomatic RO33 infection shortly before the recorded symptomatic K1 infection. Interestingly, though responding well to the conserved MSP-1_{19} and Block 2 of RO33 in both infections, donor AE7 failed to respond to Block 2 of the only PCR-detected type (K1) in 1993, and also to one of the two parasite types (MAD20) found in 1994. This lack of response to more than one of Block 2 types present in a mixed infection was the predominant pattern observed in the majority of such infections (e.g., Fig. 5, individuals D4, B6, AE7).

Table II. *Correlation between Ab levels to different Block 2 Ags*[a]

	Palo Alto	DW K1 #1	DW K1 #2	MAD20	Wellcome	DW MAD20	RO33	DW RO33	MSP-1_{19}
3D7	**0.871**	**0.846**	**0.864**	0.048	−0.108	−0.141	0.207	0.078	0.206
Palo Alto		**0.961**	**0.943**	0.007	−0.127	−0.171	0.111	−0.020	0.023
DW K1 #1			**0.932**	0.026	−0.091	−0.126	0.051	−0.066	0.034
DW K1 #2				0.015	−0.104	−0.156	0.114	−0.008	0.062
MAD20					**0.936**	**0.886**	−0.353	−0.248	0.206
Wellcome						**0.930**	−0.377	−0.236	0.196
DW MAD20							−0.380	−0.278	0.108
RO33								**0.881**	0.168
DW RO33									−0.007

[a] Correlation of anti-Block 2 Ab reactivities within and between MSP-1 Block 2 types. Ab reactivities against any one Block 2 variant antigen (measured as OD units) were compared to reactivities of all other tested antigens in a pairwise manner for sera from 39 individuals who responded to Block 2 following clinical malaria episodes in the 1993 to 1995 transmission seasons. Results are expressed as Spearman's rank correlation coefficients between antigens.

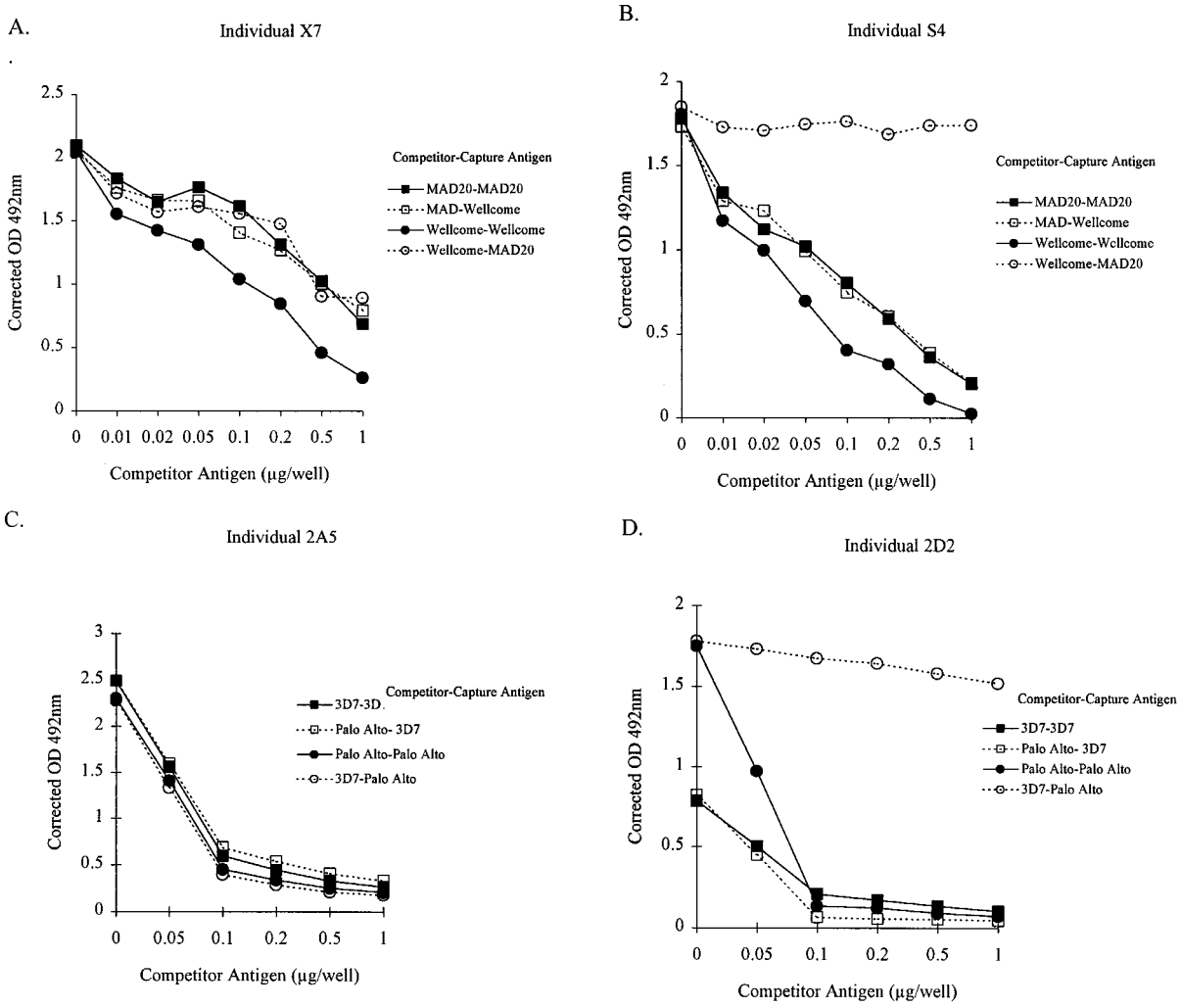


FIGURE 6. Competition ELISAs showing type and variant specificity of Abs in four individuals responding to MAD20-like or K1-like types of Block 2. Donor X7 (MAD20-type Ab, *A*) and donor 2A5 (K1-type Ab, *C*) had type-specific Abs that did not differentiate well between different variants within the types. Donor S4 (MAD20-type Ab, *B*) and donor 2D2 (K1-type Ab, *D*) had type- and variant-specific Abs. All sera were tested at 1:500 dilution. Legends indicate the pairs of competing Ags used, with the competing Ag listed first and the well-bound capture Ag second. The capture Ags were coated at 50 ng/well. The increasing amounts of competing Ag added to the diluted sera are indicated along the *x*-axis.

Type vs variant specificity of anti-Block 2 responses

Two of the three main Block 2 types (K1 and MAD20) contain numerous sequence variants but the immunologic significance of this diversity is unclear. In most malaria cases in whom IgG to the Block 2 region was detected, there was little difference between the levels of Ab reactivity with different Block 2 variants within any one type (e.g., Fig. 4, *C* and *D*). Correlation coefficients between the levels of Ab reactive with pairs of Block 2 variants within a type were all high when calculated for 39 Block 2 Ab-positive sera (Table II). This indicates that Abs to Block 2 cross-react with variants within the same type. By contrast, correlations between Ag pairs from different Block 2 sequence types (and for comparison, correlations between any Block 2 Ag and MSP-1_{19}), were insignificantly low in the same group of sera. Thus there is little or no cross-reactivity of Abs directed to the three different Block 2 types or, indeed, with MSP-1_{19}.

Competition ELISAs, using plasma samples that contained Abs of similar reactivity against all tested variants within a type, showed that Block 2-specific IgG was directed primarily against epitopes shared within a type and to a lesser extent against variant-specific epitopes (Fig. 6). Discrimination between variants within a type was revealed by these competition experiments with Abs of some but not all individuals (compare individuals S4 and X7, or 2D2 and 2A5, Fig. 6). In the case of donor S4, binding of specific Abs to the MAD20 variant of Block 2 was not inhibited by a different variant of the same type (Wellcome; see Refs. 27 and 36 for sequences of Block 2 variants). Similarly, one variant (3D7) of the K1-type Block 2 failed to inhibit binding to another variant of the same type (Palo Alto) of K1-type Abs made by donor 2D2. Such clear discrimination of variants within a type occurred in one of four high-titerd anti-MAD20 Block 2 plasmas, and five of nine K1-specific plasmas tested. Plasmas of donors X7, 2A5 (Fig. 6, *A* and *C*), and others (not shown) contained Abs that did not differentiate variants within a type. Therefore, although competition ELISA revealed Abs that are capable of discrimination between variants in some cases, IgG to the K1 and MAD20 types of Block 2 is mainly directed against shared epitopes characteristic of each type.

Duration of response to MSP-1

During the 8 to 9 mo of dry season following the summer rains, malaria transmission ceases in eastern Sudan and Daraweesh. In

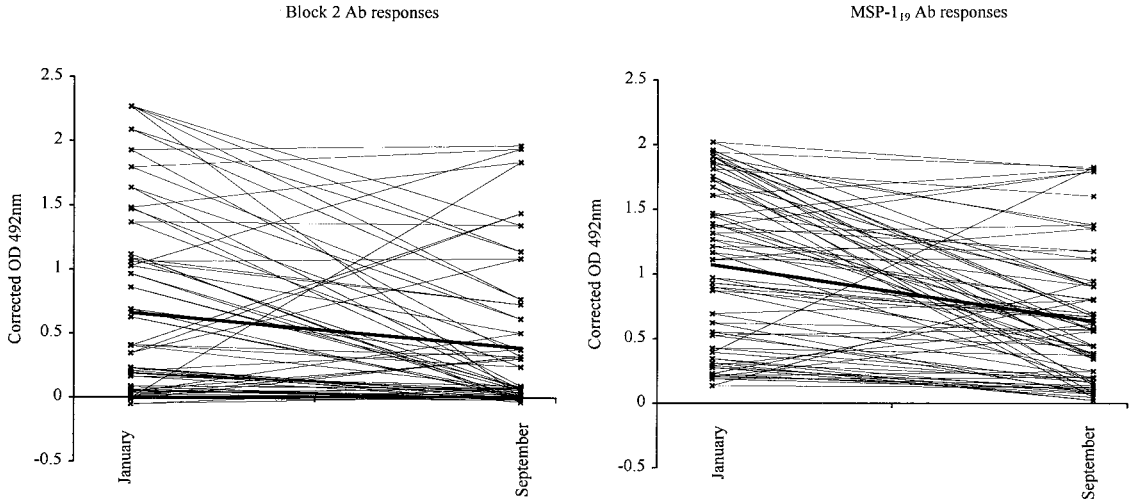


FIGURE 7. Duration of responses to C-terminal and Block 2 Ags. Ab reactivity (expressed as OD_{492}) against Block 2 Ags (*A*) and MSP-1_{19} (*B*) in 40 pairs of plasma samples from donors infected in the 1993 and 1994 transmission seasons are shown at two time points, January and September. Linear regression trend lines for each data series are shown in bold.

the absence of reinfection, Ab responses against both Block 2 and C-terminal Ags declined in most, but not all, individuals after a malaria attack. This decline is illustrated in Figure 7 for the January to September 1994 period. Mean OD levels of Abs against Block 2 declined from 0.6 to 0.36, and from mean OD of 1.04 to 0.57 for Abs against the C-terminal MSP-1_{19} Ag. After drug treatment of infections, only a few individuals maintained high Ab levels against either Block 2 or MSP-1_{19} from convalescence in January to the start of the next transmission season in September (Fig. 2, *A* and *B*). Ab responses to both the polymorphic Block 2 region and the conserved C-terminal region thus appear to be short lived, returning to low levels within a few months of parasite clearance.

Discussion

This longitudinal cohort study, which combines accurate clinical status monitoring with sensitive assays of Ab response, has revealed a more complete picture of the natural pattern of human Ab response to a major polymorphic Ag of *P. falciparum* than could be deduced from earlier cross-sectional field studies, or from experimental immunization of animals. We have used a panel of immunologically well-characterized recombinant proteins (41, 42) to measure naturally acquired Ab responses to the conserved N-terminal Block 1, the highly polymorphic Block 2, and the C-terminal 19-kDa conserved region of *P. falciparum* MSP-1 in individuals living in a Sudanese village where malaria incidence is limited to a short, well-defined transmission season. This study of 52 individuals spanning a 4-yr period shows that both the polymorphic Block 2 and the conserved C-terminal regions of the molecule were recognized by specific IgG Abs produced in response to malaria infection by most individuals. From September 1992 to September 1995, there were 77 cases of malaria in this cohort, and only two individuals did not have a malaria attack. Overall, 36 of 52 (69%) of the individuals responded to the polymorphic Block 2 region and 47 of 52 (90%) responded to the conserved C-terminal MSP-1_{19} during the study period. In contrast, few individuals (5 of 52, 10%) had IgG directed against Ags representing the conserved Block 1 that includes the peptide YSLFQKEKMVL contained in the Spf66 vaccine (28–30). This result indicates that the Block 1 sequence may be a poor immunogen and, if so, Ab responses induced by the vaccine may not be boosted by natural *P. falciparum* infections. The alternative explanation, that recombinant Block 1 Ags lack epitopes found in parasite-derived Block 1, seems to be excluded by the demonstrated ability of the Ags to induce animal Abs reactive with conserved epitopes of *P. falciparum* MSP-1 (41).

In most individuals, IgG to either the Block 2 or the C-terminal MSP-1_{19} rose only during or after a documented clinical malaria episode. This was most marked in the 1993 and 1994 transmission seasons, for which the most complete sets of pre- and postinfection plasma samples were available (Fig. 2 and Table I). In both transmission seasons, the frequency of seroreactivity against the MSP-1 Ags was significantly higher in individuals who had a clinical *P. falciparum* infection than in those who were microscopically aparasitemic and who did not suffer from malaria during the season. Interestingly, increased levels of Ab reactivity against the Block 2 and C-terminal Ags were also observed in a minority of the latter group (Table I and Fig. 1). This suggests that subclinical asymptomatic malaria infections may have occurred in these individuals. The presence of low-density parasitemia detectable by PCR helped to clarify some of these cases in which rises in IgG to MSP-1 were not preceded by clinically documented malaria. These observations indicate the existence of a higher level of acquired immunity in this population than would be expected in the context of unstable malaria transmission in the village (37), and strengthen similar conclusions of an earlier report on the prevalence of subclinical infections in this population (38).

In clinically ill and/or convalescent individuals, the frequency of IgG response to the C-terminal MSP-1_{19} was significantly higher than that against any of the Block 2 Ags tested (χ^2 test, $p < 0.01$ for both January and June samples following infection). There are several possible reasons for this difference. In a series of malaria infections in one individual, identical epitopes of the conserved C-terminal region would be presented by all parasites, and thus memory responses to MSP-1_{19} would be expected in most of the cohort members by the time of this study. However, exposure to any one type of Block 2 is less frequent. The three main types of Block 2 are distributed evenly in Daraweesh, each being present in approximately one-third of PCR-genotyped blood samples collected at several time points during this survey. For example, during the 1994 transmission season, the percentages of each type detected by PCR were 34% (MAD20), 29% (K1), and 37%

(RO33). In the 1993 and 1994 seasons, Ab responses to the three Block 2 types were also distributed equally between the three types (Table I). Thus, the probability of a reinfection with, and of memory response to, a parasite expressing the same type of Block 2 is likely to be lower than that observed to conserved epitopes. Lower still is the probability of a reinfection by and memory response to parasites of exactly the same allelic variant of Block 2.

We propose that clinical malaria episodes and their antigenic challenge are the key determinants of human responsiveness to MSP-1. Estimates of the frequency of Abs to the conserved C-terminal MSP-1 region (including MSP-1_{19}) varied from 45 to 60% in The Gambia (23, 26), to over 75% in Kenya (25), but did not reach the 90% level shown in this study. The frequencies of seroreactivity with Block 2 variants have been reported to vary between 12 and 75%, increasing with the age of donors, in Mali (34). In Burkina Faso, 21% of adults had Ab against the MAD20 variant of Block 2 (31). The frequencies of Ab responses to Block 2 in Daraweesh (69% of all donors) fall within the range detected by others, but are probably more complete, since this study used a panel of nine Block 2 Ags representing a greater variety of sequence variants compared with the more limited Ag panels available in earlier studies. The overall higher than usual frequency of Ab recognition of MSP-1_{19} in this study reflects the strategy of longitudinal sampling and close monitoring of malaria infections in these individuals over 4 yr. The lack of response to any of the MSP-1 Ags (and to other merozoite proteins, data not shown) following clinical malaria episodes in individuals C5, D6, D10, E2, and 2J4 cannot be explained, although transitory or low responses, perhaps under genetic control, are possibilities (43).

In malaria-exposed populations, the observed prevalence of Ab to malaria Ags is often less than 100%, and it has been suggested that this might reflect a genetic control of the host immune response. There is evidence indicating a regulation of humoral immune responses to a spectrum of malaria Ags by unknown genetic factors (43). The influence of MHC haplotype on immune responsiveness to epitopes from several malaria Ags including MSP-1 has been reported in mice (44–47), but few associations have been found between HLA types and high or low responsiveness to defined *P. falciparum* Ags (48–53). Since Ab responsiveness has been measured by a single cross-sectional survey in most studies, and the relationship of response to proven malaria infection was often unknown, the frequency of nonresponsiveness to MSP-1 in humans has probably been overestimated.

It has also been suggested that nonresponsiveness of some individuals to certain malaria Ags may be due to the phenomenon of "clonal imprinting," or "original antigenic sin." This hypothesis suggests that an individual's B cell repertoire against parasite Ags might become fixed by his or her first or early exposure to a particular parasite antigenic variant, thereby preventing the recognition of other variant Ags in subsequent infections (53, 54). Analysis of Ab responses to MSP-1 in the cohort of this study does not support this hypothesis. Although there is a very strong tendency to produce Ab to the conserved MSP-1_{19} part of the protein, over a period of years and several genetically distinct clinical malaria infections, individuals do not show a fixation of their Ab response to any particular MSP-1 Block 2 type. Of the individuals followed in this study, 25% produced IgG to more than one Block 2 type, and 69% recognized at least one Block 2 type over the 4 yr of sampling. PCR typing of the Block 2 of parasites present at or before the time of the malaria episode facilitated the comparison of Block 2 types of infecting parasites with the specificity of anti-Block 2 responses produced by the same individual. The specificity of Ab responses matched the Block 2 type of the infecting parasites in the majority of cases. Specificities of Abs to the Block 2 region were therefore indicative of the most recent infection episode and not fixed on previously experienced parasite variants. It is likely that the low frequencies of Abs to the Block 2 region are due to low exposure to malaria combined with antigenic polymorphism of this region of MSP-1, rather than to the hosts' innate inability to recognize the region, or nonrecognition due to clonal imprinting.

Why then is there an overall lower frequency of responsiveness to the polymorphic Block 2 region compared with the conserved MSP-1_{19}? A possible reason is that Ab responses to Block 2 were so transitory that plasma samples collected 2 to 3 mo after infection did not contain detectable IgG specific for that infection. Supporting this argument is a sharp decline in anti-Block 2 IgG levels evident even in many individuals who did respond to the region (Fig. 5, *C* and *D*; Fig. 7). We have also tested the possibility that those patients who had no detectable IgG to Block 2, had anti-Block 2 IgM, but have found no specific IgM in nine such individuals (data not shown). Alternatively, variant-specific (rather than type-specific) Abs may have been present but were not detected due to the limited number of variants in our panel of Ags. High correlation of "within-type" recognition of Block 2 variants derived from *MSP-1* alleles of parasites obtained from Daraweesh patients and of variants derived from non-Sudanese reference alleles (Table II), argues against a high frequency of narrowly specific Ab recognition of single Block 2 variants, rather than recognition of all variants within a type. However, variant-specific Abs clearly do exist and the possibility of single variant-specific responses cannot be excluded, since it is not known to what extent the recombinant Ags used in this study represent the full range of allelic variants in Block 2 of the parasite population in the village. It is also true that a proportion of individuals tested did not respond to our Block 2 Ags at any point in the study.

An influential hypothesis has proposed that repetitive epitopes in malaria Ags are immunodominant, eliciting T cell-independent production of ineffective Abs and thus "immuno-evasion" (55). The extensive allelic diversity in the Block 2 region of MSP-1, including the RO33 type of Block 2 that contains no repeat sequences, does not support any role in immune evasion (56). In most cases where Abs to Block 2 of the two repetitive types (K1 type and MAD20 type) were detected, it was notable that they cross-reacted with all tested variants within these types. Of 77 documented malaria episodes, only 4 plasma samples from 4 patients had Abs that unambiguously discriminated between Block 2 variants within the K1-like or the MAD20-like types. The strong correlation between Ab levels to variants within a type (Table II) indicates that Abs to all types of Block 2 are directed predominantly against epitopes that are shared within a type rather than against the repetitive sequences that differ between variants. The lower frequency of variant-specific IgG vs type-specific IgG seen in our cohort argues that repetitive sequences in Block 2 are not immunodominant in this Ag.

Our observations that Ab responses to MSP-1 are short lived and correspond to recent malaria episodes support the conclusions of earlier studies (31, 32). The rapid decline of anti-MSP1 responses after the removal of parasites by drug treatment resulted in low or undetectable levels of anti-MSP-1 IgG in many individuals by the start of the next malaria season (Fig. 7). If Abs to MSP-1 contribute to antiparasite immunity, such immunity could be rapidly lost in people in such circumstances of short seasonal transmission and/or effective antimalarial treatment. However, there is no evidence that Ab responses to Block 2 correlate with increased risk of infection, as proposed by Tolle et al. (34), since patients

with responses to Block 2 Ags were no more likely to be subsequently infected than those without such responses (Fig. 2). Our results support the view that Ab to the Block 2 region is a specific marker of recent infection, rather than a prognostic indicator of susceptibility to disease. None of the earlier studies related specific Ab responses to the polymorphic Block 2 region directly to parasite MSP-1 genotypes, since only single antigenic variants were used as detection Ags, and no conclusions could be drawn about variant- or type-specific responses to MSP-1 in defined *P. falciparum* infections. By using PCR detection and typing of infecting parasites, the close correlation between individuals' type-specific Ab responses and the genotype of the parasites infecting each host is clarified. The importance of coordinated analysis of the parasites' Ag(s) together with response to them in individual hosts is clarified by longitudinal sampling regimes such as this. This methodology has permitted a more realistic estimation of the overall frequency of human responsiveness to defined regions of MSP-1, previously underestimated by cross-sectional studies.

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