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Genetic Diversity and Antigenic Polymorphism in *Plasmodium falciparum*: Extensive Serological Cross-Reactivity between Allelic Variants of Merozoite Surface Protein 2

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Diversity in the surface antigens of malaria parasites is generally assumed to be a mechanism for immune evasion, but there is little direct evidence that this leads to evasion of protective immunity. Here we show that alleles of the highly polymorphic merozoite surface protein 2 (MSP-2) can be grouped (within the known dimorphic families) into distinct serogroups; variants within a serogroup show extensive serological cross-reactivity. Cross-reactive epitopes are immunodominant, and responses to them may be boosted at the expense of responses to novel epitopes (original antigenic sin). The data imply that immune selection explains only some of the diversity in the *msp-2* gene and that MSP-2 vaccines may need to include only a subset of the known variants in order to induce pan-reactive antibodies.

Allelic diversity in malaria parasites is widely assumed to be a mechanism for immune evasion (7, 24). Among surface proteins, diversity arises from multiple point mutations or variations in the numbers, lengths, and sequences of amino acid repeats. Repeat diversity may arise from intragenic recombination, misalignment of repeated DNA sequences (6), or complementary-strand slippage (12, 13) during DNA replication. The AT-rich nature of the *Plasmodium falciparum* genome may facilitate the generation of new variants (28). The maintenance of variants in the population implies that they have a selective advantage (e.g., evasion of preexisting immune responses) or that the mutations are selectively neutral and that population diversification occurs randomly.

In some genes (e.g., the circumsporozoite surface protein [*csp*] gene), point mutations cluster in areas immunodominant for T cells (16) and can lead to loss of epitope recognition (38) or antagonism of T-cell responses (26), although it is not clear that either of these leads to the avoidance of protective immunity. Similarly, monospecific antibodies differentiate between allelic variants of merozoite surface proteins (13, 23), but the human antibody response is commonly directed at conserved or semiconserved epitopes (8, 34) and direct evidence for the evasion of antibody-dependent immunity is lacking.

Comparison of the frequencies of nonsynonymous-to-synonymous mutations (20) for *P. falciparum* genes provides evidence of diversifying selection in *msp-1* (11), which encodes merozoite surface protein-1, but the data are less clear for *msp-2*, which encodes merozoite surface protein-2 (9, 11, 21), and the significance of the findings is uncertain, given the limitations of the method (37). Comparison of intra- and interpopulation variances in allele frequencies provides evidence for selection on a polymorphic region of *msp-1* (5) and dimorphic regions of *msp-2* (4), but there are no data for polymorphic regions of *msp-2*. Comparison of inter- and intraspecific levels of variation suggests balancing selection on the erythrocyte-binding antigen EBA-175 (25) and the apical membrane antigen AMA-1 (22). However, except for *msp-1*, for which selection may be mediated by strain-specific antibodies (5), the nature of the selective forces is not clear.

msp-2 is the most polymorphic locus yet described for *P. falciparum* (14), with over 170 alleles sequenced to date (18). *msp-2* alleles are divided into two families (IC-1-like and FC27-like) (31) based on dimorphic sequences internal to the conserved N and C termini (Fig. 1). Point mutations occur in all regions of the gene (including conserved and dimorphic sequences), and variations in sequence, length, and number of amino acid repeats occur in the polymorphic region. Seroepidemiological studies indicate that the conserved N and C termini of the molecule are poorly recognized by immune serum but that the dimorphic sequences are strongly recognized by most adult immune sera (34). Analysis of responses to polymorphic sequences is difficult, as it is rarely known with which parasite genotypes an individual has been infected and to which sequences he or she might be expected to have made antibodies. Nevertheless, sera from a high proportion of both children and adults do recognize recombinant proteins representing individual polymorphic sequences (34), indicating ei-

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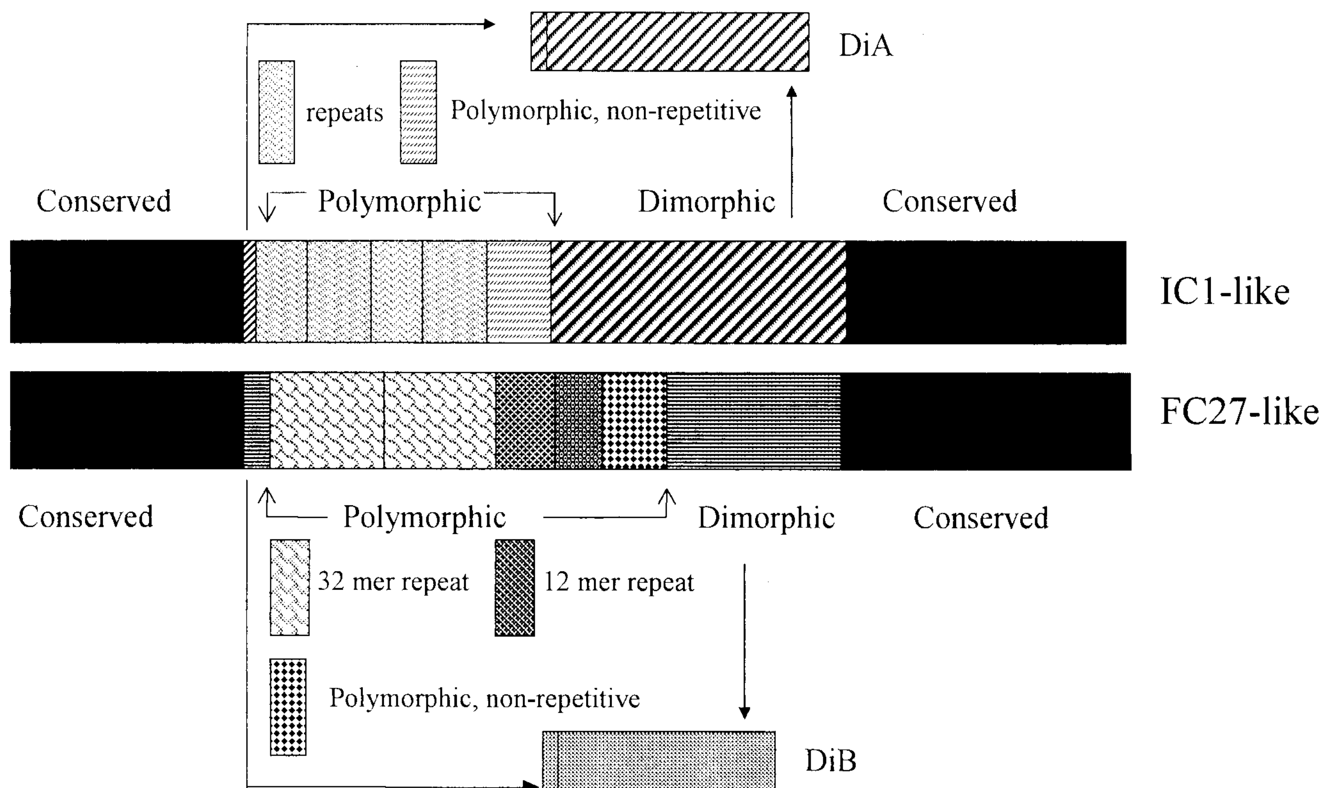

FIG. 1. Schematic representation of MSP-2. Highly conserved 5′ and 3′ sequences define the *msp-2* locus; point mutations within these regions may indicate immune selection at the level of T-helper epitopes (12). Dimorphic families are defined by sequences internal to the MSP-2 defining sequences; the type sequence for family A is IC-1 and for family B is FC27 (31). The central region of the protein comprises amino acid sequences repeated in tandem (with varied sequences and numbers of amino acids) and a nonrepetitive sequence that is highly polymorphic in the IC-1 family but less so in the FC27 family. The regions incorporated into the dimorphic IC-1-like (DiA) and dimorphic FC27-like (DiB) recombinant proteins are shown.

ther that these sequences are common in the parasite population or that they contain epitopes that cross-react with other sequences. There is good evidence that anti-MSP-2 antibodies contribute to protective immunity. In independent epidemiological studies, immunoglobulin G3 (IgG3) antibodies have been associated with resistance to clinical malaria (2, 32), anti-MSP-2 antibodies have inhibited the growth of parasites in vitro (10), and in a recent human trial, immunization with the 3D7 variant of MSP-2 prevented reinfection with parasites carrying alleles of the same family (IC-1) (15).

These findings presented us with a paradox. MSP-2 is highly polymorphic, and antibodies to it appear to be protective, but there is little evidence that these antibodies impose selective pressure on the most polymorphic regions of the gene. A potential explanation is that diversifying selection operating on repetitive MSP-2 sequences may not be detectable by current population genetic methods. Alternatively, antibodies to MSP-2 may be nonprotective or antibodies may be cross-reactive and unable to differentiate between parasites of different *msp-2* genotypes and therefore be unable to exert selective pressure.

The aim of this study was to determine whether sequence differences between MSP-2 variants are indicative of functional antigenic polymorphism at the level of antibody responses and thus represent a potential mechanism of immune evasion or whether serological cross-reactivity between MSP-2 variants reduces the degree of true antigenic diversity.

MATERIALS AND METHODS

Study design. The study area, the village of Prampram on the south coast of Ghana, and the study design have been described elsewhere (35). *P. falciparum* transmission is perennial, stable, and of moderate intensity (5 to 10 infectious bites per person per year) (1). After informed consent was obtained from mothers, a blood sample was obtained from them and their children at delivery and from the children every 4 weeks. Asymptomatic infections were not treated, and infants did not receive malaria prophylaxis. If the child was unwell, a blood film was examined immediately and antimalarial chemotherapy was instituted if necessary. Informed consent was obtained from all the participants in the study or from their guardians. Ethical approval was obtained from the ethical review committee of the University of Edinburgh; the institutional review board of the Noguchi Memorial Institute of Medical Research, University of Ghana; and the Ghanaian Ministry of Health.

Parasite detection and genotyping. Giemsa-stained blood films were examined by oil immersion microscopy. PCR genotyping of *msp-1* and *msp-2* was performed for all *P. falciparum*-positive samples, and the polymorphic domains of *msp-2* were sequenced as described previously (14). Sequences were confirmed by analysis of multiple clones (at least five) and/or repeated PCR amplification and sequencing. Each unique sequence was designated with the child's identification number and the age of the child (in weeks) when that sequence was first isolated (e.g., 5-34 is the sequence isolated from child 5 at 34 weeks of age).

Recombinant proteins. The polymorphic sequences of the various MSP-2 alleles were amplified by PCR by using a 5′ primer (CGCGGATCCAATATG AGTATAAGGAGAAGTATG) (the *Bam*HI restriction site and the 5′ extension are underlined) that anneals the extreme 3′ end of the N-terminal conserved

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TABLE 1. Sequence alignment of MSP-2 IC1-like alleles[a]

Group	Allele	Sequence
Group 1	29-18	AESKPST GA(GGSA)$_6$~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDG~~~~~~~ N~GADAEGSSSTPAT~~ TTTT KTTTTTTTTN
	29-58	AKSKPST GA(GGSA)$_6$~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDG~~~~~~~ N~GADAEGSSSTPAT~~ TTTT KTTTTTTTTN
	65-58	TESNPPA GAGASG NPPAGAGASG SAGASG NPPAGAGASG SAGASG SAGASG SAG ~~~~~~~~~~~~~~~ AEGSSSTPAT~~~~~~~ TTTTTTTN
	52-10	AESNLPT GAGARS (GDGARS)3 ~~~~~~~~~~~~~~~~~~~~~~~GDGAVASA GSG ~~~~~~~~~~~ ADAEGSSSTPAT PATTTTTTTTTTTTTTN
	15-66/52-38*	AVSNPST(GAGGSGSA)$_6$~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTTKTTTTTTTTN
	71-34	AVSNPST GAGGSGSA~~~~~GGSGSA GAGGSGSA ~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTKTTTTTTTTN
Group 2a	17-34	AESKTPT GSGAGASGSAGSGAGASGSA~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTTKTTTTTTTTN
	17-18	AESKTPT GSGAGASGSAGSGAGASGNA~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTTKTTTTTTTTN
	47-66	AESKTPT GSGAGASGSAGSGVGASGSA~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTTKTTTTTTTTN
	15-34	AESKTPT GSGAGASGSAGSGAGASGSA~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPSADAEGSSSTPAT TTTTKTTTTTTTTN
	65-38	AESKTPT GSGAGASGSA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT TTTTKTTTTTTTTN
Group 2b-2a hybrid	15-70	TESKPPT GD~~GAGASGRAGA~ GASGRAGSGA~~ GASGSA~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSTSTPAT TTTTKTTTTTTTTN
Group 2b	62-46	TESNPPT GTGTGAGASGRAGAGAGASGRAGAGAGA(GASGRA)$_3$GAGAGSGDGAVASA GSGDGAVVSAGNGANPGADAEGSSSTPAT PATTTTTTTTTTTN
	62-50	TESNPPT GTGTGAGASGRAGAGAGASGRAGAGAGA(GASGRA)$_3$GASAGSGDGAVASA GSGDGAVASAGNGANPGANAEGSSSTPAT PATTTTTTTTTTTN
	62-622	TESNPPT GTGTGAGASGRAGAGAGASGRAGAGAGA(GASGRA)$_3$GASAGSGDGAVASA GSGDGAVASAGNGANPGADAEGSSSTPAT PATTTTTTTTTTTN
	62-623	TESNPPT GTGTGAGASGRAGAGA~~~~~~~~~~ (GASGRA)$_3$GASAGSGDGAVASA GSGDGAVASAGNGANPGADAEGSSSTPAT PATTTTTTTTTTTN
	62-422	AESKPST ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GASAGSGDGAVASA GSGDGAVASAGNGANPGADAEGSSSTPAT PATTTTTTTTTT~N
	62-621	EESKPPT GASGSAGS~~~~~~ GSGAVTSAGNGANPSADAKRSPSTPATPATPATPAT~~~~~~~~~~~~~~~~ TTTTTTTN
	29-22	EESKPPT GASGSAGS~~~~~~ GSGAVASAGNGANPSADAKRSPSTPATPATPATPAT~~~~~~~~~~~~~~~~ TTTTTTTN
	120-10	TESKPPT GD~~GAGS~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GAGAVASAGNGANTGADAKKSPSTPAT~~~~~~ ~~~~~~~~~~~~~~~~~TTTTTTTN
Group 3	62-14	EESKPPT GASGSAGS~~~~~~ GSGAVASAGNGANPGADAERSPSTPATPATPATPATPATPATPATPAT~ ~~~TTTTTTTTTTN
	62-18	KESKPPT GASGSAGS~~~~~~ GSGAVASAGNGANPGADAERSPSTPATPATPATPAT~~~~~~~~~~~~~~~~ TTTTTTTN
	62-22	KESKPPT GASGSAGS~~~~~~ GSGAVASAGNGANPGADAERSPSTPATPATPATPATPATPATPATPATPATPATTTTTTTTTTTTN
	47-26	EESNLPT GAGAVAGSGAGAVASSGAGAVA(GSGAVA)8 GSGAGAGNGA~~~~~~~~~ ~~~~~~~GNGANPGADAKRSPSTPATTTTTTTTN
Group 4	7-302	EESNLPT GAGAVAGSGAGAVASSGAGAVA(GSGAVA)3 GSGAGAGNGA~~~~~~~~~ ~~~~~~~GNGANPGADAKTSPSTPATTTTTTTTN
	47-30a	EESNLPT GAGAVA~~~~~~~~~~~~~~~~~~~~~~ GSGAGAGNGA~~~~~~~~~ ~~~~~~~GNGANPGADAKRSPSTPATTTTTTTTN
	71-38	EESNPPT VAGA~~GSGAGAVAGSGAGAVA GSGAGAVA ~~~~~~~~ GAGAGA ~~~ GSG~~~~~~ ANPGADAKKSPSTPATTTTTTTTN
Group 5	47-50	AVSNPST(GAGGSGSA)$_6$ GAGASGSGSA GASGSGSA GAGAGAGASGSA ~~~~~~~ GSG ~~~~~~~~~~~ADAKRSPSTPATTTTTTTTTTTN
	45-50	AESNPST GA(GGSA)$_6$ GGNA (GGSA)$_{12}$ ~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGN~~~~ GANPGANAKKSPSTPAT ~~TTTTTTTN
IC-1/FC27 Hybrid	71-38R	TESRTPT GAVASA GSGAGSGAVASAGSGAVASAGSGAVASGS~~~~~~~~~~ FC27 type

[a] The colors highlight regions of sequence variation and similarity between alleles, including single amino acid substitutions and tandem amino acid repeats. *, Sequences of 15-66 and 52-38 (52-A2) are identical.

region and two 3′ primers (GGGAATTC**TTA**GGTACTGGTAGATGCTTCTGCATC and GGGAATTC**TTA**TTCACTCTCTTCTCCTTTACCGTC) (the *Eco*RI restriction site and the 5′ extension are underlined; the stop codons are in bold) that anneal the extreme 5′ end of the dimorphic segment of the IC-1 and FC27 sequences, respectively. The PCR products were purified using a High Pure PCR product purification kit (HPPPK; Boehringer Mannheim), double digested with *Bam*HI and *Eco*RI, and inserted into linearized pGEX-2T vectors (Amersham Pharmacia Biotech, Uppsala, Sweden). Recombinant proteins were expressed in *Escherichia coli* by using the glutathione *S*-transferase (GST) gene fusion system (Amersham Pharmacia Biotech) and were purified by using glutathione-Sepharose (34). The resulting proteins consisted of full-length GST, a hexapeptide linker sequence (Leu-Val-Pro-Arg-Gly-Ser), the short N-terminal MSP-2 dimorphic region, the entire MSP-2 polymorphic region, and 8 or 12 amino acid (aa) residues from the C-terminal MSP-2 dimorphic region of IC-1 or FC27, respectively. Based on the alignments of Fenton et al. (13), all of the proteins start at residue 44 and end at residue 109 (in the 3D7 sequence) or residue 154 (in the FC27 sequence). The exact MSP-2 sequences in the expressed proteins are shown in Tables 1 and 2. All of the proteins were soluble in phosphate-buffered saline. Unfused GST, including the hexapeptide linker sequence, was expressed from native pGEX-2T vectors and used as a control in enzyme-linked immunosorbent assays (ELISAs).

The IC-1 and FC27 dimorphic proteins (DiA and DiB) represent the N- and C-terminal dimorphic sequences with the central polymorphic sequences removed by splicing by overlap extension (19). The N- and C-terminal conserved and dimorphic segments of the MSP-2 gene were amplified by using genomic DNA from *P. falciparum* lines 3D7 (IC-1-like) and HB3 (FC27-like) with the following primer pairs: for the 3D7 N-terminal sequence, 5′GAAGGTAATTAAAACATTGTC and 3′TGCTTCTGCATCAGTAGAAGGCTTACTTTC; for the 3D7 C-terminal sequence, 5′GATGCAGAAGCATCTCTACC and 3′AATGAAGTAAATATAGCAACACATTC; for the FC27 N-terminal sequence, 5′GAAGGTAATTAAAACATTGTC and 3′TCCTTTACCGTCTTTTGGAGCATTTGCACC; and for the FC27 C-terminal sequence, 5′GACGGTAAAGGAGAAGAG and 3′AATGAAAGTAAATATAGCAACACATTC.

The primers were designed such that one primer in the N-terminal amplifica-

TABLE 2. Sequence alignment of 12 MSP-2 FC27-like alleles[a]

Subgroup	Allele	Sequence
subgroup 1a	47-34	ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA (ADTIASGSQRSTNSASTSTTNNGESQTTTPTA)$_2$ ADTPTAT ~~~~~~~~~ ESSSSGNAPNKT
	47-30b	ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA (ADTIASGSQRS/GTNSASTSTTNNGESQTTTPTA)$_2$ ADTPTAT ~~~~~~~~~ ESSSSGNAPNKT
	5-34	ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA ADTIASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT ESNSPSPPITTT ESSSSGNAPNKT
subgroup 1b	62-14	ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA ADTIASRSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT ESNSPSPPITTT ESSSSGNAPNKT
	62-22	ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA AGTIASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT EGNSPSPPITTT ESSSSGNAPNKT
subgroup 2	65-62	ANKGSNTNSVGANAPN ~~~~~~~~~~~~~~~~~~~~~~~~~~~ (ADTIASGSQRSTNSASTSTTNNGESQTTTPTA)$_3$ ADTPTAT KSNSPSPPITTT ESSSSGNAPNKT
	15-34	ANEGSNTNSVGAN~~~ ~~~~~~~~~~~~~~~~~~~~~~~~~~~ (ADTIASGSQRSTNSASTSTTNNGESQTTTPTA)$_3$ ADTPTAT ~~~~~~~~~ESSSSGNAPNKT
	65-54	ANESSNTNRVGANAPK ADT IASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT ESNSPSPPITTT KSNSPSPPITTT (KSNSPSPPITTT)$_2$ ~~~~~~~~ESSSSGNAPNKT
subgroup 3	5-38	ANEGSNTKSVGANAPK ADT IASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT ESNSPSPPITTT ESNSPSPPITTT KSNSPSPPITTT ~~~~~~~~ESSSSGNAPNKT
	120-10	ANEGSNTNRVDANAPK ADT IASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT ESNSRSPPITTT ESNSRSPPITTT (ESNSRSPPITTT)$_2$ ~~~~~~~ ESSSSGNAPNKT
	5-26	ANESSNTNRVVANAPK ADTVASGSQRSTNSASTSTTNNGESQTTTPTA ADTPTAT KSNSPSPPITTT ESNSPSPPITTT (KSNSPSPPITTT)$_2$ ~~~~~~~~ESSSSGNAPNKT
recombinant	71-38	IC1 type~~~~~~~~~~~~~~ASGSQSSTNSASTSTTNNGESQTTTPTA ADTPTAT KSNSPSPPITTT ESNSPSPPITTT ~~~~~~~~~~~~~~~~~ ESSSSGNAPNKT

[a] The letters in red indicate single amino acid differences between sequences.

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52-10 AESNLPT GAGARSGDGARSGDGARSGDGAR~ SGDGAVASAGSG~~~~~~ ADAEGSSSTPATPATTTTTTTTTTTTTTN
52-38 A**V**SN**PS**T (GAGGSGSA)$_6$~~~~~~~~~~~~~~~~~~~GSGDGAVASA**RN**GANPGADAEGSSSTPAT ~~~TTTT**K**TTTTTTTTN

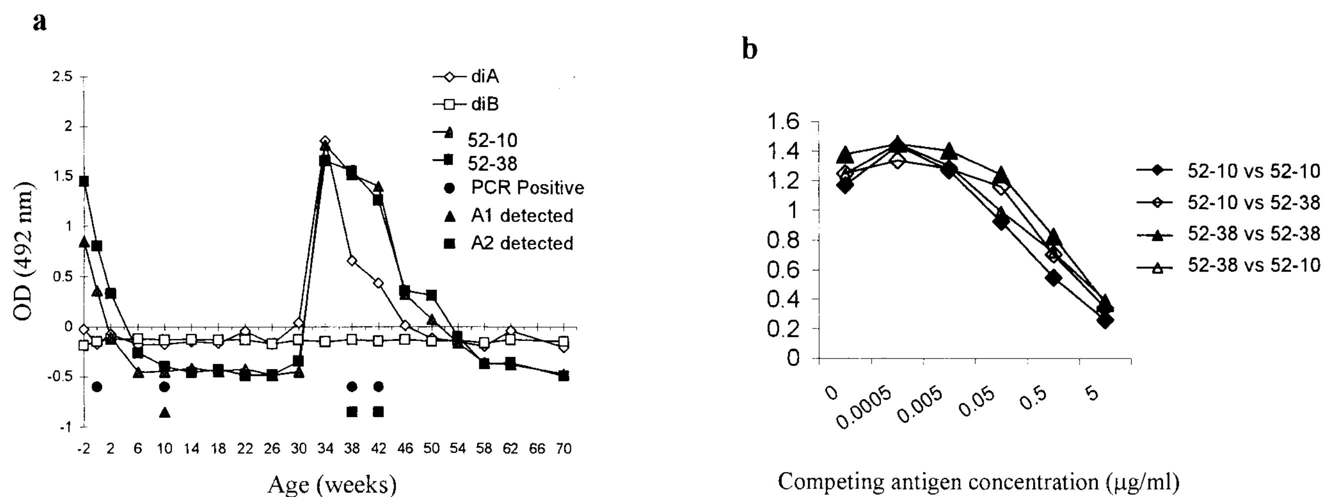

FIG. 2. Cross-reactivity of antibodies to MSP-2 proteins 52-10 1 (clone A1) and 52-38 2 (clone A2). (a) The IgG response over the first 18 months of life of child 52 is shown. DiA and DiB are dimorphic sequences of the IC-1 (DiA) and FC27 (DiB) families. The blood sample obtained at −2 weeks of age was collected from the mother prior to delivery. (b) Competition ELISA for child 52. The first-named protein indicates the protein on the plate; the second is the protein with which the serum was preincubated. The boldface type in the amino acid sequences identifies sequence differences between the two proteins.

tion pair had a 5′ extension (underlined) that was homologous to a sequence in the C-terminal amplification pair (underlined), allowing hybridization of the two PCR products in the second round of PCR. The first-round PCR products were purified with HPPPK. Equal parts of the N- and C-terminal PCR products were used as templates for a second round of PCR using primers for the 3′ extreme-N-terminal (CGCGGATCCAATATGAGTATAAGGAGAAGTATG) and 5′ extreme-C-terminal (GGGAATTCTTAAGATTGTAATTCGGGGGA) conserved sequences of the MSP-2 molecule. The primers incorporated *Bam*HI or *Eco*RI endonuclease restriction sites (underlined) for cloning purposes. As these primers were conserved, the same primer pair could be used for the amplification of both 3D7 and HB3. The resulting PCR products were cloned and expressed in pGEX-2T as described above. The expressed proteins were soluble and comprised the complete N- and C-terminal dimorphic sequences of either 3D7 (IC-1-like) or HB3 (FC27-like), but lack the polymorphic or conserved sequences, and were fused to GST and the hexapeptide linker sequence.

Recombinant GST-MSP-1_{19} was produced as described previously (8).

All of the recombinant vectors were sequenced to confirm that they each contained a full-length insert and that the insert was in frame. All of the proteins were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bacterial contamination was minimal; all of the proteins were of the expected molecular mass, indicating that full-length proteins had been expressed. All of the proteins appeared to be stable when they were stored at −80 or −20°C.

Serology. ELISAs were conducted exactly as described previously (34). Except where stated to the contrary, sera were tested at a dilution of 1:1,000. The optical density (OD) for the GST control protein was subtracted from the OD for the fusion protein to provide a corrected OD. Corrected-OD values are given for the results shown in all figures except where otherwise stated. A positive control serum (a pool of hyperimmune serum from Gambian adults) was used in all of the assays to confirm that recombinant proteins retained their antigenicity and their ability to bind to the plate.

Nucleotide sequence accession numbers. The MSP-2 gene sequences have been deposited in GenBank (accession numbers AF217004 to AF217043).

RESULTS

Cloning and sequencing of *msp-2* alleles. The *msp-2* alleles in 71 *P. falciparum* isolates obtained from 13 Ghanaian children were sequenced (14). Alleles encoding 41 different proteins were obtained; 29 sequences were IC-1-like, and 11 were FC27-like; one sequence was a hybrid (IC-1-like N terminus and FC27-like C terminus).

Prior to any immunological analysis, the IC-1-like sequences were divided into five groups based on sequence similarities in the nonrepetitive C terminus. IC-1 group 2 (IC-1-2) was subdivided (into 2a and 2b) on the basis of differences in the N-terminal and repeat sequences (Table 1). IC-1 group 5 (IC-1-5) contains two sequences that do not fit clearly into any of the other groups. The FC27 sequences were less diverse and were grouped into three rather similar groups (Table 2). FC27 group 1 was subdivided (into 1a and 1b) according to the presence or absence of the ESNSPSPPITTT sequence.

Fifteen alleles, selected to represent different sequence groups, were expressed as soluble recombinant proteins.

Cross-reactive antibodies recognize multiple IC-1-like variants of MSP-2. Plasma samples from individual children were tested for IgG reactivity with recombinant proteins derived from parasites with which they were known to have been infected. We noticed apparent serological cross-reactivity between proteins of differing sequence; an example is shown in Fig. 2a. Child 52 was asymptomatically infected with *P. falciparum* at 10, 34, and 38 weeks of age. Parasite densities were extremely low, being detectable by PCR but not by microscopy (14); two parasite clones (A1 and A2), with different *msp-2* genotypes, were identified. Serial plasma samples were tested for reactivity with recombinant proteins representing these two MSP-2 sequences. Reactivities with variant 52-10 (IC-1-1) (first isolated when the child was 10 weeks of age) and those with variant 52-38 (IC-1-2a) (isolated when the child was 38

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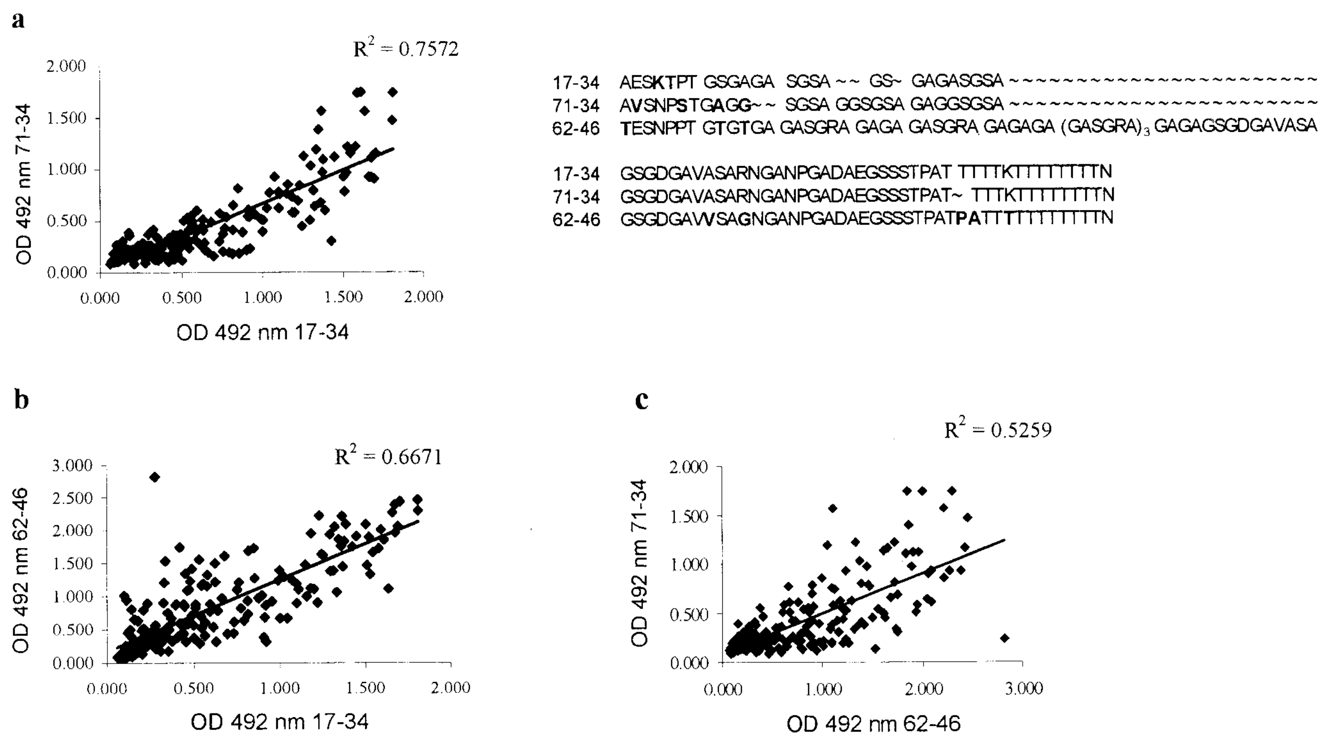

FIG. 3. Identification of cross-reactive epitopes in the IC-1-like family of MSP-2. The amino acid sequences of three MSP-2 proteins and the correlation of antibody responses of 201 Gambian sera to proteins 17-34 and 71-34 (a), proteins 17-34 and 62-46 (b), and proteins 62-46 and 71-34 (c) are shown. The boldface type in the amino acid sequences identifies sequence differences between the proteins.

weeks of age) were identical over a period of more than 1 year. Antibodies in the mother's plasma that were also present in the child until 4 weeks of age also recognized both antigens, although ODs were higher for 52-38 (A2) than for 52-10 (A1). The two proteins clearly differed in sequence (Fig. 2) but shared regions of identity at the C terminus and within the repeats.

To confirm that antibodies to the two MSP-2 variants were cross-reactive, we performed competition assays. Preincubation of the child's serum with 5 μg of protein 52-10 (IC-1-1) per ml completely absorbed antibodies binding to 52-38 (IC-1-2a) and vice versa (Fig. 2b).

Cross-reactivity of antibodies to IC-1-like variants is a general phenomenon. To determine whether cross-reactive recognition of MSP-2 variants is a general feature of the human antibody response, we tested plasma samples from 201 malaria-exposed Gambians (aged 3 to 70 years) (30, 34) for reactivity to nine different IC-1-like proteins and, as a control, to a recombinant MSP-1_{19} protein, which has no obvious homology to MSP-2. The responses to MSP-2 and MSP-1_{19} were correlated, although the correlation coefficients were low ($r^2 \leq 0.33$); this presumably reflects concomitant exposure to both antigens during natural infection. In contrast, the correlation of antibody responses to some pairs of IC-1-like MSP-2 proteins was very high (Fig. 3 and Table 3). The highest r^2 values were seen within group 2, notably for two proteins (17-34 and 71-34) with homology in their repeat sequences and an identical 30-aa sequence at the C terminus. Group 2 was divided into subgroups 2a and 2b on the basis of point mutations in the nonrepetitive N and C termini and differences in the repeat motifs; correlations within group 2a were higher than correlations between group 2a and a group 2b protein (62-46).

To confirm that population-level correlations reflect cross-reactivity (rather than concomitant exposure to non-cross-reactive proteins), sera with high titers of antibody to IC-1-2a proteins were tested in competition assays (Fig. 4). For serum 1578 (Fig. 4a and b), maximal binding to 15-66 was lower than to 17-34 or 65-38, but all three proteins completely blocked binding to each other, indicating recognition of a conserved or cross-reactive epitope. Similar results were obtained for numerous other sera, including serum 1638 (Fig. 4c and d); in this case antibodies bound to 15-66 with slightly higher affinity than to the other two proteins. Proteins 15-66, 17-34, and 65-38 all belong to the same sequence group (IC-1-2a) and are identical in their C-terminal nonrepeated sequences; the cross-reactive epitope may lie in this region. However, 17-34 and 65-38 also share a 17-aa sequence at the N terminus of the polymorphic region; multiple sequence differences in this region of protein 15-66 may affect the avidity of antibody-antigen interactions. Serum 1634 did not differentiate between group 2a proteins 17-34 and 71-34 (Fig. 4e) or 15-66 (data not shown) but recognized protein 47-50 (group 5) with somewhat lower avidity (Fig. 4f). The fact that 47-50 (IC-1-5) competes for binding with group 2a proteins suggests that the cross-reactive epitope lies towards the N terminus or at the extreme C terminus of the protein.

Variant 15-70 has the C-terminal nonrepetitive sequence of group 2a but the N-terminal sequence and GASGRA repeat

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TABLE 3. Correlation of antibody responses to pairs of IC-1-like MSP-2 alleles

Protein[a]	Subgroup	Correlation with[b]:							
		29-18	15-66	71-34	17-34	65-38	15-70*	62-46	47-26
29-18	1								
15-66	2a	0.3722							
71-34	2a	0.3242	**0.7239**						
17-34	2a	0.4401	**0.7825**	**0.7572**					
65-38	2a	0.2609	**0.6967**	**0.5530**	**0.7294**				
15-70	2a	0.4345	**0.8113**	**0.6951**	**0.8190**	**0.6968**			
62-46	2b	0.4851	0.5818	0.5259	0.6671	0.4888	0.6411		
47-26	4	0.4535	0.3064	0.2978	0.3140	0.1889	0.3369	0.3462	
47-50	5	0.5501	0.5140	0.3840	0.4337	0.3357	0.5124	0.4545	0.4974

[a] We tested 201 Gambian sera for all proteins, except proteins 15-70 and 47-50, for which we tested 189 sera.

[b] Boldface numbers indicate results for alleles within putative serogroup IC1-2a; underlined numbers indicate results of a comparison of putative serogroups IC1-2a and IC1-2b. All correlation coefficients are statistically significant ($P < 0.00001$). A comparison of responses to MSP-2 proteins with responses to an antigenically unrelated MSP-1 protein gave r^2 values of approximately 0.33 (data not shown).

motif of group 2b. As responses to 15-70 are more closely correlated with group 2a proteins than with the group 2b protein (Table 3), the commonly recognized cross-reactive epitope may lie towards the C terminus of the polymorphic domain.

Cross-reactivity within the FC27 family of MSP-2. Sequence diversity is less extensive in the FC27 family than in the IC-1-like family, and cross-reactivity between variants would thus be less surprising. The 32-mer and 12-mer repeats vary in number (12-mer repeats being completely absent in some sequences), and minor sequence differences occur within the repeats (Table 2). The number of 12-mer repeats can affect the avidity of antibody binding (27). All alleles shown in Table 2 share a conserved 12-aa sequence (ESSSSGNAPNKT) at the C terminus and a conserved 7-aa sequence (ADTPTAT) between the 32-mer and 12-mer repeats. Either or both of these sequences may confer cross-reactivity and were included in all of our recombinant proteins.

We screened sera from three children with antibodies to at least one of the FC27-like variants for reactivity to proteins representing different sequence groups of FC27-like alleles. All three sera recognized variants 47-30b, 62-22, and 5-34; cross-reactivity was confirmed by competition assay (Fig. 5 a to c) and by correlation analysis (Fig. 5d). The responses to all three variants were highly correlated (Table 4), confirming that they belong to the same serogroup (FC27 group 1 in Table 2). Although we had subdivided group 1 on the basis of sequence data (Table 2), the serological data suggested that the three variants actually belong to a single serogroup and that the ESNSPSPPITTT sequence does not contribute to antibody specificity.

Although the children's sera recognized the FC27 group 1 proteins 47-30b, 62-22, and 5-34, none of them recognized FC27 group 3 variant 5-38 or 120-10 (data not shown). In the panel of immune sera, responses to group 1 proteins were very poorly correlated with responses to group 3 proteins (Table 4 and Fig. 6a and b). This poor correlation is perhaps not surprising given the extent of the sequence differences between group 1 and group 3 (Table 2).

The antibody responses to group 3 proteins 5-38 and 120-10 (which differ by 3 aa in the N-terminal region and in the number of 12-mer repeats) were very poorly correlated with each other (Fig. 6c). This was principally due to sera binding to 5-38 with much lower OD values than for 120-10; a few sera that gave high responses to 5-38 and 120-10 distorted this relationship (Fig. 6c).

DISCUSSION

msp-2 is one of the most polymorphic loci yet described for *P. falciparum*, and it is widely assumed that polymorphism at antigenic loci provides a selective advantage for the parasite, allowing it to evade preexisting antibody or T-cell responses. This study has demonstrated that while some sequence polymorphisms in *msp-2* do lead to antigenic polymorphism, many genetically disparate MSP-2 proteins are serologically cross-reactive. Cross-reactivity occurs for variants within the same dimorphic family, both within and between sequence-defined subgroups. Importantly, the conserved or cross-reactive epitopes are immunodominant, such that the majority of antibodies binding to epitopes within the polymorphic domains recognize cross-reactive rather than allele-specific epitopes (as shown by complete blocking in competition assays). Somewhat surprisingly, cross-reactivity between the sequence groups was most evident for the IC-1-like variants where sequence diversity is most pronounced. The ability of diverse IC-1-like MSP-2 antigens to induce cross-reactive antibodies was confirmed by mouse immunizations (data not shown but), where a single polymorphic protein was able to induce antibodies to at least three other variants within the same family. In contrast, for the FC27-like sequences, where sequence polymorphisms tend to be limited to variation in the number of repeats, cross-reactivity was evident only within sequence-defined serogroups and there was very little evidence of cross-reactivity between groups.

For FC27 variants, the sharing of conserved ADTPAT and ESSSSGNAPNKT sequences did not confer cross-reactivity, which indicates that these sequences are not the target of dominant antibody responses. Point mutations in the N-terminal, nonrepetitive region of the polymorphic domain did not appear to have a major effect on antibody binding, as synthetic peptides representing three different sequences were able to compete with each other for antibody binding (data not shown). The major serological subdivision of the FC27-like alleles was between those that contain multiple 32-mer repeats

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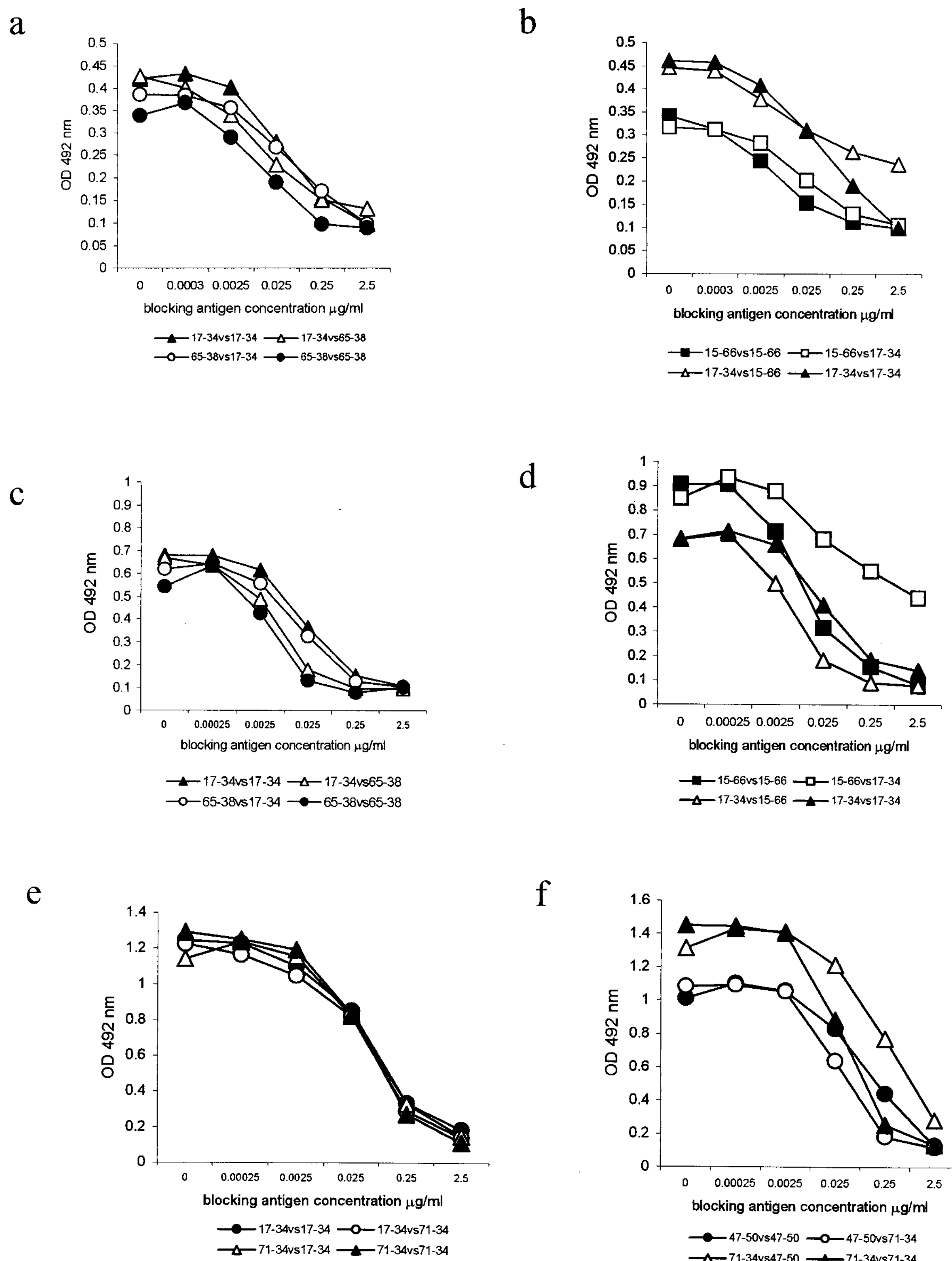

47-50 AVSNPST(GAGGSGSA)$_6$ GAGASGSGSA GASGSGSA GAGAGAGASGSAGSG ~~~~~~~~~~~ ADAKRSPSTPATTTT~~ TTTTTTTN
15-66 AVSNPST(GAGGSGSA)$_6$~~~~~~~~~~~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPATTTTTKTTTTTTTTN
71-34 AVSNPST GAGGSGSA~~~~~GGSGSA GAGGSGSA ~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPAT~TTTKTTTTTTTTN
17-34 AESK**T**PT GSGAGASGSAGSGAGASGSA~~~~~~~~~~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPATTTTTKTTTTTTTTN
65-38 AESK**T**PT GSGAGASGSA~~~~~~~~~~~~~~~~ ~~~~~~~~~ GSGDGAVASARNGANPGADAEGSSSTPATTTTTKTTTTTTTTN

FIG. 4. Cross-reactivity within IC-1 subgroup 2 alleles. Competition ELISA results for individual sera from malaria-exposed individuals against pairs of MSP-2 proteins are shown. In each case, the first-named protein was used to coat the ELISA plate and the second-named protein was preincubated with the serum. (a and b) Serum 1578; (c and d) serum 1638; (e and f) serum 1634. The boldface type in the amino acid sequences identifies sequence differences between the proteins.

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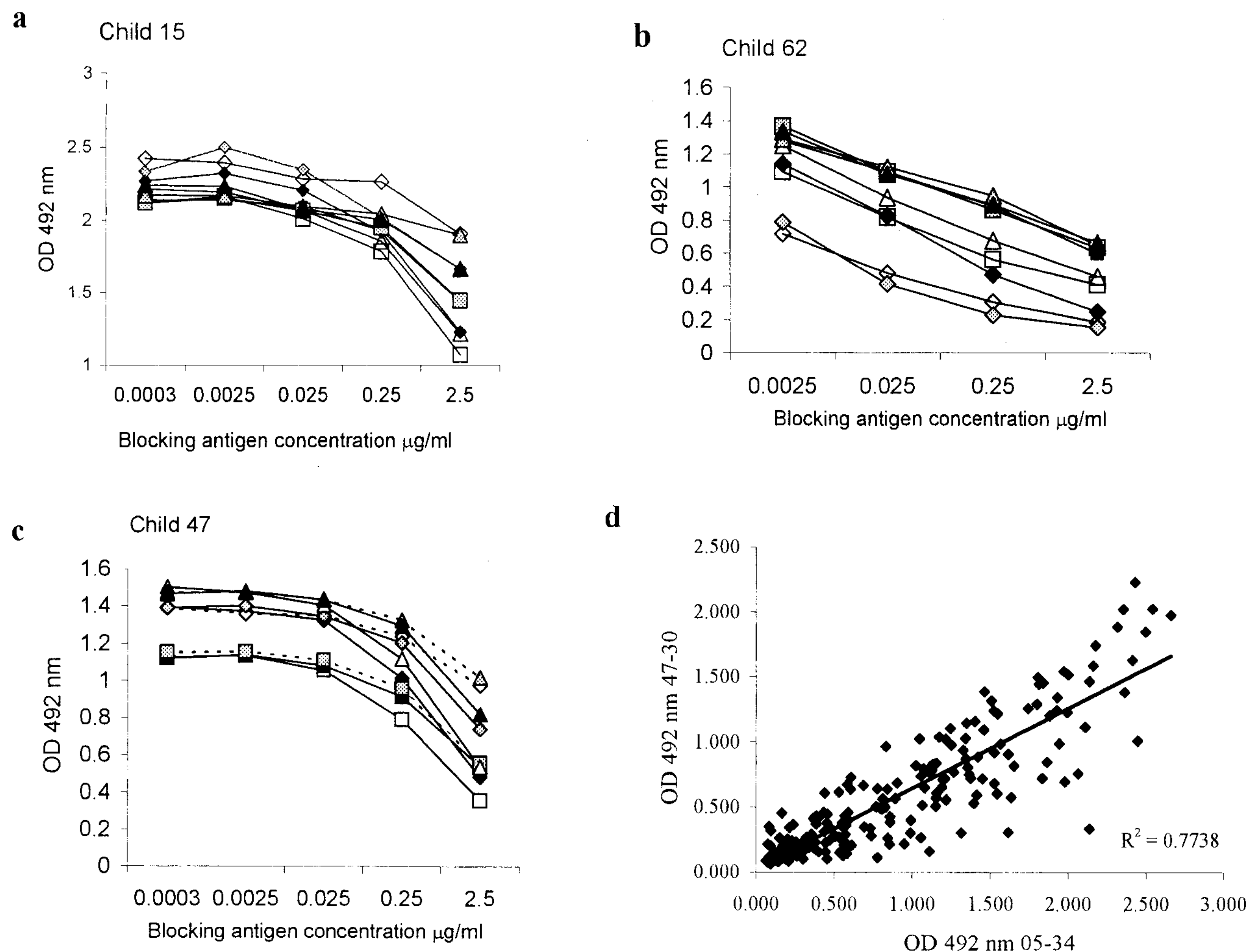

FIG. 5. Cross-reactive antibody responses to group 1 FC27-like proteins. (a to c) Competition ELISA results for sera from three different Ghanaian children. ▲, 5-34 versus 5-34; △, 5-34 versus 47-30; ◬, 5-34 versus 62-22; ■, 62-22 versus 62-22; □, 62-22 versus 47-30; ▩, 62-22 versus 5-34; and ◆, 47-30 versus 47-30; ◇, 47-30 versus 62-22; ◈, 47-30 versus 5-34. In each case, the first-named protein was used to coat the ELISA plate and the second-named protein was preincubated with the serum. (d) Correlation of antibody-binding responses (ODs) of 201 individual sera from malaria-exposed donors to proteins 47-30 and 5-34.

and single (or no) 12-mer repeats (groups 1 and 2 in Table 2) and those that contain a single 32-mer repeat and multiple 12-mer repeats (group 3). Within group 3, differences in the number of 12-mer repeats seriously affected the avidity of antibody binding, confirming our previous observations (27)

TABLE 4. Correlation of antibody responses to pairs of FC27-like MSP-2 alleles[a]

Protein	Subgroup	Correlation with[b]:				
		47-30	5-34	62-22	15-34	5-38
47-30	1a					
5-34	1b	**0.776**				
62-22	1b	**0.606**	**0.771**			
15-34	2	0.004	0.013	0.006		
5-38	3	0.001	0.0003	0.052	0.013	
120-10	3	0.084	0.103	0.109	0.0002	0.093

[a] Results shown are for 201 Gambian serum samples.
[b] The values in bold are for the alleles within putative serogroup FC27-1.

These data provide evidence in support of the immune selection hypothesis for the FC27 family of *msp-2* alleles. However, the number of "strains" or serogroups appears to be rather limited (perhaps as few as 2 or 3), with each serogroup comprising multiple cross-reactive variants. It remains to be seen whether the gradual acquisition of antibody responses to different FC27 serogroups correlates with the development of protective immunity (i.e., whether these antibodies are of functional importance, for example, in inhibiting parasite growth) or whether antibodies to FC27-specific epitopes can structure *P. falciparum* populations into discrete, nonoverlapping strains (17).

For the IC-1-like alleles, homology in the nonrepetitive C terminus of the polymorphic domain predicts cross-reactivity at the population level and defines at least four separate serogroups. However, some sera appear to recognize cross-reactive epitopes within the repetitive sequence such that cross-reactivity is observed between as well as within sequence groups. It

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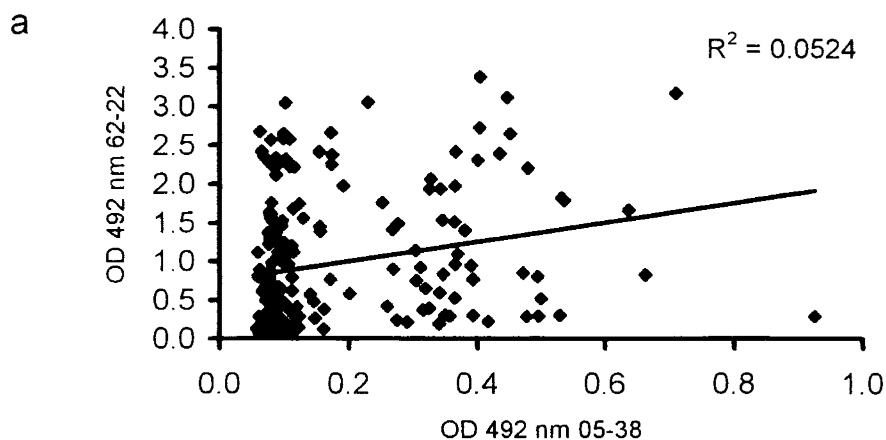
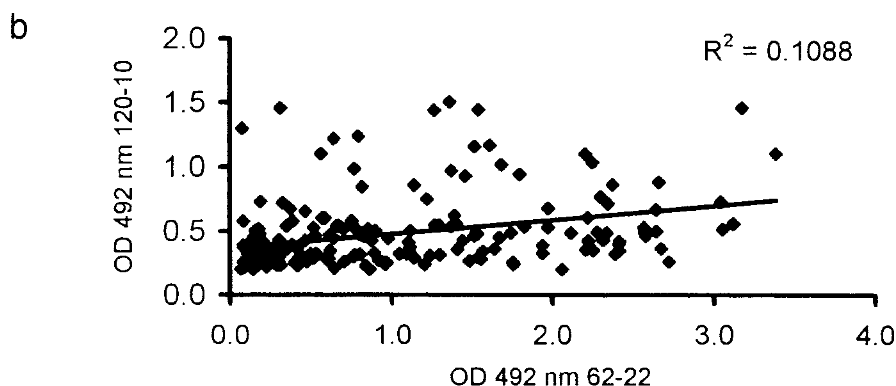
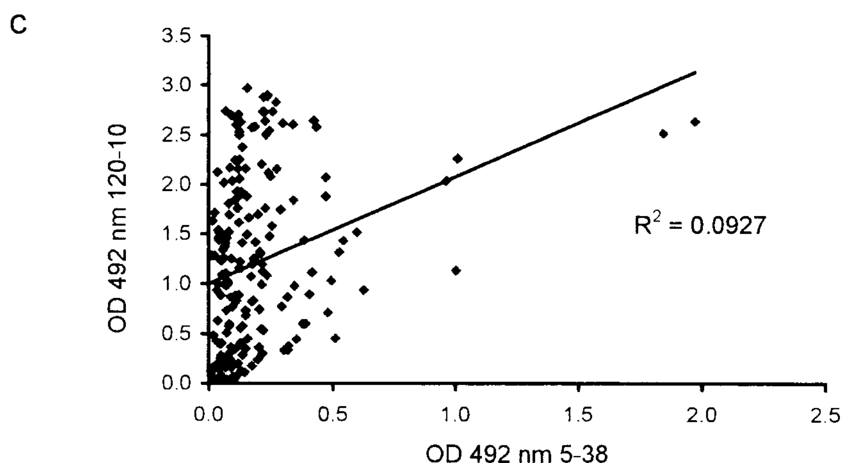
a



b



c



62-22 ANEGST TNSVDANAPK ADTVARVSQSSTNSASTSTTNNGESQTTTPTA AGTIASGSQRSTNSASTSTTNNGESQTTTPTA
5-38 ANEGSNTKSVGANAPK ADT IASGSQRSTNSASTSTTNNGESQTTTPTA ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
120-10 ANEGSNTNRVDANAPK ADT IASGSQRSTNSASTSTTNNGESQTTTPTA ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

62-22 ADTPTAT EGNSPSPPITTT ~~~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~ ~~~~~~~~~~ ESSSSGNAPNKT
5-38 ADTPTAT ESNSPSPPITTT ESNSPSPPITTT ESNSPSPPITTT KSNSPSPPITTT ~~~~~~~~~~ ESSSSGNAPNKT
120-10 ADTPTAT ESNSRSPPITTT ESNSRSPPITTT ESNSRSPPITTT ESNSRSPPITTT ESNSRSPPITTT ESSSSGNAPNKT

FIG. 6. Lack of cross-reactivity between FC27 subgroup 1 proteins and other FC27-like proteins. (a to c) Correlation of antibody-binding responses (ODs) of 201 individual sera from malaria-exposed donors to proteins 62-22 and 5-38 (a); proteins 120-10 and 62-22 (b); and proteins 120-10 and 5-38 (c).

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is thus likely that there is a continuum of cross-reactivity within the IC-1 family. It is much less clear that allelic polymorphism might lead to immune evasion within this family of *msp-2* alleles, but we cannot rule it out. Differential binding of individual sera to cross-reactive proteins indicates that the major effect of sequence variation may be modifying the affinity or avidity of antibody-antigen interactions rather than leading to complete abolition of antibody binding sites. Further studies are required to determine whether these affinity or avidity differences are sufficient to favor parasite survival, as has been proposed previously (3). Furthermore, it is necessary to establish whether these cross-reactive antibodies can actually mediate cross-strain immunity—i.e., that they have a protective role in inhibiting parasite invasion or growth—or whether, despite their extensive cross-reactivity, they are in fact irrelevant in terms of protection. The limited data available from MSP-2 vaccination trials suggest that immune responses induced by one member of a dimorphic family do in fact protect against many variants within that family but do not protect against variants from the other family (15).

We have previously reported that repeated infection with genetically distinct parasites leads to boosting of the existing antibody response rather than to generation of novel specificities (33); a recent study of anti-MSP-2 responses in Vietnamese people reports a similar phenomenon (36). We suggested that this may be an example of original antigenic sin (29). The same phenomenon may explain the tendency of the response to MSP-2 to focus primarily on cross-reactive epitopes. If these epitopes have a natural tendency (because of their physical location or tertiary structure) to be immunogenic for B cells, antibodies to them will predominate during a primary immune response and, once formed, will focus attention on similar (cross-reactive) epitopes during subsequent infections. If so, the MSP-2 sequence of a primary *P. falciparum* infection may determine the pattern of antibody reactivity to future infections and may account in part for individual heterogeneity in antibody specificity (29). Original antigenic sin may be beneficial to the host, allowing the immune system to ignore rare epitopes and to concentrate on epitopes present on the majority of invading parasites. Alternatively, if immunodominant or cross-reactive epitopes are not protective, original antigenic sin may favor the parasite by maintaining high levels of nonprotective antibodies and protecting more vulnerable epitopes.

These ideas leave the significance of the extensive polymorphism of MSP-2 unresolved. If many (or most) polymorphisms do not confer antigenic specificity and are thus not a mechanism for immune evasion, what is their purpose? One explanation may be that much of the polymorphism is due to random (and neutral) diversification of the population but that occasional mutations generate novel serogroups that are subject to immune selection. Immune selection implies that anti-MSP-2 antibodies can either prevent infection or reduce transmission of *P. falciparum*. Repeated reinfection in the presence of cross-reactive anti-MSP-2 antibodies in the cohort of Ghanaian children (22) suggests that anti-MSP-2 antibodies do not confer complete protection from infection, but the very low parasite densities seen in such children may indicate that anti-MSP-2 antibodies are able to limit parasite replication, which may have implications for transmission.

In summary, this study has revealed an extensive serological cross-reactivity of genetically distinct variants within the two dimorphic families of MSP-2. Within a family, variants can be grouped into a small number (two or three) of serogroups on the basis of recognition by cross-reactive antibodies. The data imply that the antigenic polymorphism of MSP-2 may be less extensive than anticipated from DNA sequences and strengthen the argument for investigation of MSP-2 as a realistic target for vaccine-induced immunity.

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