INFECTION AND IMMUNITY, Feb. 1995, p. 456–466
0019-9567/95/$04.00+0

Vol. 63, No. 2

# Serum Antibodies from Malaria-Exposed People Recognize Conserved Epitopes Formed by the Two Epidermal Growth Factor Motifs of $MSP1_{19}$, the Carboxy-Terminal Fragment of the Major Merozoite Surface Protein of *Plasmodium falciparum*

ANDREA F. EGAN,[1] JONATHAN A. CHAPPEL,[2] PETRA A. BURGHAUS,[2] JOANNE S. MORRIS,[3] JANA S. McBRIDE,[1] ANTHONY A. HOLDER,[2] DAVID C. KASLOW,[4] AND ELEANOR M. RILEY[1]*

*Institute of Cell, Animal and Population Biology, Division of Biological Sciences, University of Edinburgh, Edinburgh,[1] Division of Parasitology, National Institute for Medical Research, Mill Hill, London,[2] and Department of Epidemiology and Population Sciences, London School of Hygiene and Tropical Medicine, London,[3] United Kingdom, and Laboratory for Malaria Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland[4]*

Received 18 July 1994/Returned for modification 24 September 1994/Accepted 11 November 1994

**The major merozoite surface protein of *Plasmodium falciparum* (PfMSP1) is a candidate antigen for a malaria vaccine. A 19-kDa C-terminal processing product of PfMSP1 ($PfMSP1_{19}$) is composed of two domains sharing a cysteine-rich motif with epidermal growth factor (EGF) and is the target of monoclonal antibodies which block erythrocyte invasion in vitro. We have evaluated human antibody responses to $PfMSP1_{19}$ by using recombinant proteins representing the EGF motifs encoded by the two main alleles of the *MSP1* gene. We find that both EGF motifs are antigenic but that only 10 to 20% of malaria-exposed individuals have serum antibodies that recognized either of the motifs. When both EGF motifs were expressed together as a single protein, they were recognized by more than 40% of sera from malaria-exposed individuals. Major epitopes recognized by human antibodies are dependent upon the correct tertiary structure of the protein and are cross-reactive between the different allelic sequences of $PfMSP1_{19}$. This suggests that antibodies induced by vaccination with one or the other allelic forms of the protein could recognize all strains of *P. falciparum*. Immunoglobulin G (IgG) subclass-specific enzyme immunoassays indicate that $PfMSP1_{19}$ antibodies are predominantly of the IgG1 subclass.**

Clinical symptoms of malaria are associated with the asexual multiplication of merozoites within erythrocytes. A prophylactic malaria vaccine would need to block this stage of the parasite's life cycle. The major merozoite surface protein-1 (16, 19, 21) of *Plasmodium falciparum* (PfMSP1) is considered an important vaccine candidate because monoclonal antibodies (MAbs) against it can block erythrocyte invasion in vitro (1, 11, 26, 30, 32) and primates immunized with either purified native PfMSP1 or recombinant proteins or synthetic peptides representing parts of the protein are partially or completely protected against experimental infections with *P. falciparum* (9, 13, 16, 17, 28, 29, 33).

PfMSP1 is the precursor to several major surface proteins of the merozoite (4, 14, 18, 26). After synthesis as a single protein, PfMSP1 is processed to produce four major fragments ($PfMSP1_{83}$, $PfMSP1_{28}$, $PfMSP1_{38}$, and $PfMSP1_{42}$). At the time of merozoite release, the $PfMSP1_{42}$ fragment undergoes secondary processing to form a 33-kDa ($PfMSP1_{33}$) product, which is shed, and a 19-kDa fragment ($PfMSP1_{19}$), which remains on the merozoite surface during erythrocyte invasion and is present on ring stage parasites (1). It has been suggested that this secondary processing of $PfMSP1_{42}$ to $PfMSP1_{19}$ is a prerequisite for erythrocyte invasion (1, 2, 4). The C-terminal fragments of PfMSP1 are of particular interest with respect to vaccine development, since naturally acquired antibodies to $PfMSP1_{42}$ are associated with resistance to clinical malaria in Gambian children (31) and MAbs mapping to the $PfMSP1_{19}$ fragment inhibit merozoite invasion in vitro (1, 11, 26, 30). In addition, an MAb recognizing an epitope within the C terminus of the PfMSP1 homolog of *Plasmodium yoelii* (PyMSP1) is able to passively protect mice against this malaria infection (6).

$PfMSP1_{19}$ consists of two domains, each with six highly conserved cysteine residues which are characteristic of epidermal growth factor (EGF) motifs (3, 10). The primary sequence of $PfMSP1_{19}$ is highly conserved except for four amino acid residues that are subject to dimorphic substitutions (22, 27). In the Wellcome sequence, these four residues are Q in the first motif and K-N-G in the second motif, while in the MAD20 sequence, they are E and T-S-R, respectively (Fig. 1).

In this study, we have evaluated the serological recognition of recombinant proteins representing the double and separate EGF motifs of $PfMSP1_{19}$ by antibodies from people living in a malaria-endemic area. We conclude that the major epitopes within $PfMSP1_{19}$ that are recognized by human antibodies are conformation dependent and require the presence of both EGF motifs. Furthermore, we report that human antibodies recognize epitopes which are conserved, or cross-reactive, between the two commonly occurring allelic forms of $PfMSP1_{19}$ and are mainly of the immunoglobulin G1 (IgG1) subclass.

* Corresponding author. Mailing address: Institute of Cell, Animal and Population Biology, Division of Biological Sciences, Ashworth Laboratory, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland. Phone: (44)31-650-5540. Fax: (44)31-667-3210. Electronic mail address: eriley@ed.ac.uk.

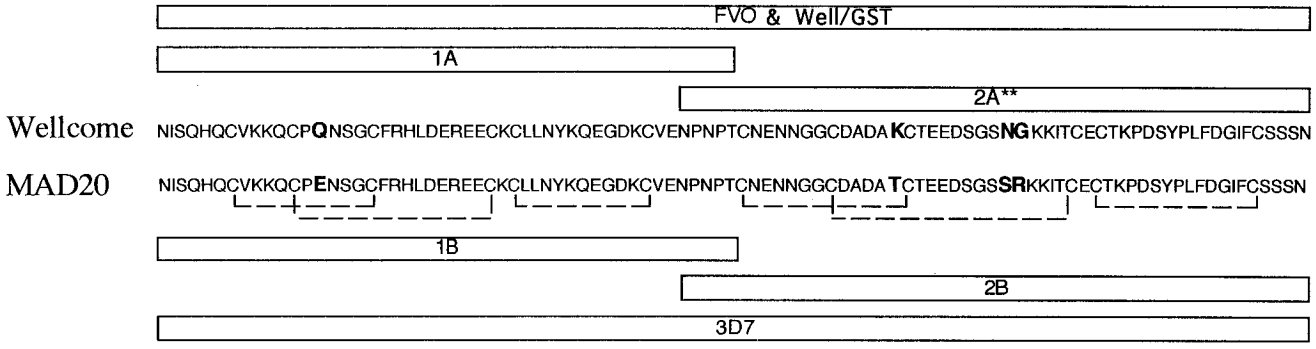


FIG. 1. Partial sequences of the Wellcome and MAD20 allelic forms of $PfMSP1_{19}$, showing the positions of sequence dimorphism, putative disulfide bonds (3, 22), and the recombinant proteins used. Dimorphic amino acids are shown in boldface type; putative positions of disulfide bonds are indicated by dashed lines. Proteins 1A, 2A, FVO, and Well/GST represent the Wellcome allelic sequence; proteins 1B, 2B, and 3D7 represent the MAD20 sequence. The first EGF motif is represented by 1A and 1B; the second motif is represented by 2A and 2B. FVO (Wellcome $MSP1_{19}$), 3D7, and Well/GST represent both EGF motifs of $PfMSP1_{19}$. FVO and Well/GST have the same $PfMSP1_{19}$ sequence but differ in the expression system used to produce them (*S. cerevisiae* and *E. coli*, respectively). The protein FVO/E represents a recombinant form of $PfMSP1_{19}$ with the first motif of MAD20 and the second motif of Wellcome (MAD/Well $MSP1_{19}$) (not shown). **Protein 2A was found to be insoluble and thus was not used in ELISAs.

## MATERIALS AND METHODS

**Recombinant PfMSP1 proteins.** Primary sequences of the recombinant proteins are shown in Fig. 1. Single first and second EGF motifs of the two allelic prototypes of PfMSP1, represented by the MAD20 and Wellcome isolates of *P. falciparum* (27), and the double EGF motif of the Wellcome form were expressed as glutathione *S*-transferase (GST) fusion proteins (34) in *Escherichia coli* transformed with recombinant pGEX3 plasmids (8). As a control, GST was produced by using the pGEX3 plasmid without any insert. The double EGF motif and three of the four single EGF motif constructs were expressed as soluble fusion proteins. The second EGF motif of the Wellcome allele (2A in Fig. 1) was insoluble in aqueous solution and only slightly soluble in 8 M urea and thus was not used. All the GST fusion proteins were recognized by conformation-dependent MAbs, indicating that the proteins assumed an approximately native conformation, with correctly formed disulfide bonds (5, 26).

Proteins representing three different versions of $PfMSP1_{19}$ were produced in recombinant *Saccharomyces cerevisiae* (23), the MAD20 and Wellcome allelic prototypes (3D7 and FVO) and a recombinant form (MAD20 first EGF motif with Wellcome second EGF motif [FVO/E]; see Fig. 1). The addition of a histidine tag to the C terminus of these fusion proteins enabled them to be purified on nickel-nitrilo-triacetic acid-agarose (23).

The purity of the fusion proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein yield was estimated by comparison with bovine serum albumin standards.

**Human sera.** Blood was collected from people living in rural areas close to the town of Farafenni in the Gambia. Malaria transmission in this area is seasonally endemic, with new infections occurring during and immediately following the rainy season (July to December) (15). Serum samples were obtained at the end of the rainy season (October) from 195 children and adults (aged 1 to 70 years) and were stored at −20°C until used. Prior exposure to malaria was confirmed by serology; all sera were positive (by immunofluorescence) for antibodies to malaria schizonts, with titers ranging from 1:160 to >1:20,000. Control serum samples were obtained from 28 European adults and 14 European children with no previous exposure to malaria.

**MAbs.** The following $PfMSP1_{19}$-specific murine MAbs were used: 7.5, 12.8, and 12.10 (26); 111.2 and 111.4 (20); 6E2/53 and 4H4/34 (11) (a kind gift of A. Saul, Queensland Institute of Medical Research, Brisbane, Australia); and 14-1C (a kind gift of R. Reese, Agouron Institute, La Jolla, Calif.).

**Immunoassays. (i) ELISA.** Antibodies reacting with recombinant PfMSP1 proteins were detected by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Immulon 4; Dynatech, Billingshurst, United Kingdom) were coated overnight at 4°C with proteins diluted in carbonate buffer (0.015 M $Na_2CO_3$, 0.035 M $NaHCO_3$ [pH 9.5]). The saturating concentration of protein, determined by titration, was from 0.1 to 5 μg/ml, depending on the antigen. Plates were washed three times in phosphate-buffered saline (PBS, pH 7.2)-Tween 20, blocked with a 1% solution of nonfat powdered milk in PBS-Tween 20 (blocking buffer), and washed again. Then 100 μl of serum or MAb was diluted in blocking buffer, incubated at room temperature for 5 h, added to duplicate wells, and incubated overnight at 4°C. Optimal dilutions of serum or MAbs were determined by titration. For all human sera, the steepest slope of the titration curve was observed at concentrations between 1:1,000 and 1:10,000 (data not shown). The optimum concentration of human serum for the assay was thus selected as 1:3,000 (24).

After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (100 μl of 1:5,000) or HRP-conjugated rabbit anti-mouse IgG (100 μl of 1:1,000) (both from Dako Ltd., High Wycombe, United Kingdom) were added to the plates, incubated for 3 h at room temperature, and developed with $H_2O_2$ and *o*-phenylenediamine (Sigma, Poole, United Kingdom) for 10 min at 4°C. The reaction was stopped by the addition of 20 μl of 2 M $H_2SO_4$. Plates were read at 492 nm. For GST fusion proteins, the optical density (OD) value of the GST control was subtracted from the OD of GST recombinant fusion proteins to give a specific OD (ΔOD) for the response to the PfMSP1 antigens. For yeast-derived proteins, when no fusion protein was involved, the original OD values are shown.

**(ii) Competition ELISA.** To determine whether antibodies recognizing individual recombinant proteins also recognized (cross-reacted with) other recombinant proteins, competition ELISAs were performed. The test serum was diluted in blocking buffer to which was added recombinant protein at a range of concentrations. After incubation for 5 h at room temperature, the blocked serum was added to immunoplates coated with a second recombinant protein. The remainder of the assay was performed as above.

Competition ELISAs were also performed to determine whether epitopes recognized by human antibodies were similar to those recognized by murine MAbs. To see if human antibodies could inhibit binding of the MAbs, plates coated with an antigen were first incubated with various concentrations of human serum. After extensive washing, a dilution of an MAb was added to the plate, incubated for 3 h, and developed with HRP-anti-mouse IgG and *o*-phenylenediamine. The reciprocal experiment was also performed to see if MAbs could inhibit binding of the human sera. In this case, antigen-coated plates were first

TABLE 1. Prevalence of antibodies to $PfMSP1_{19}$ in the Gambia

| Age group | No. | No. (%) of sera recognizing recombinant protein[a]: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Wellcome 1st motif | MAD20 1st motif | MAD20 2nd motif | MAD20 $MSP1_{19}$ | MAD/Wellcome $MSP1_{19}$ | Wellcome $MSP1_{19}$ | Wellcome/GST $MSP1_{19}$ |
| Children (<8 yr) | 33 | 5 (15.2) | 3 (9.1) | 6 (18.2) | 4 (12.1) | 7 (21.2) | 7 (21.2) | 11 (33.3) |
| Adolescents (9–15 yr) | 60 | 8 (13.3) | 11 (18.3) | 5 (8.3) | 12 (20) | 22 (36.7) | 20 (33.3) | 25 (41.7) |
| Adults (>16 yr) | 92 | 19 (20.7) | 19 (20.7) | 13 (14.1) | 38 (43.1) | 53 (57.6) | 50 (54.3) | 43 (46.7) |
| Total | 195 | 32 (16.4) | 33 (16.9) | 24 (12.3) | 54 (27.6) | 82 (42.1) | 77 (39.5) | 79 (40.5) |

[a] A positive response is one which is greater than the mean plus 2 SDS of the OD values for 42 control (malaria nonexposed) sera.

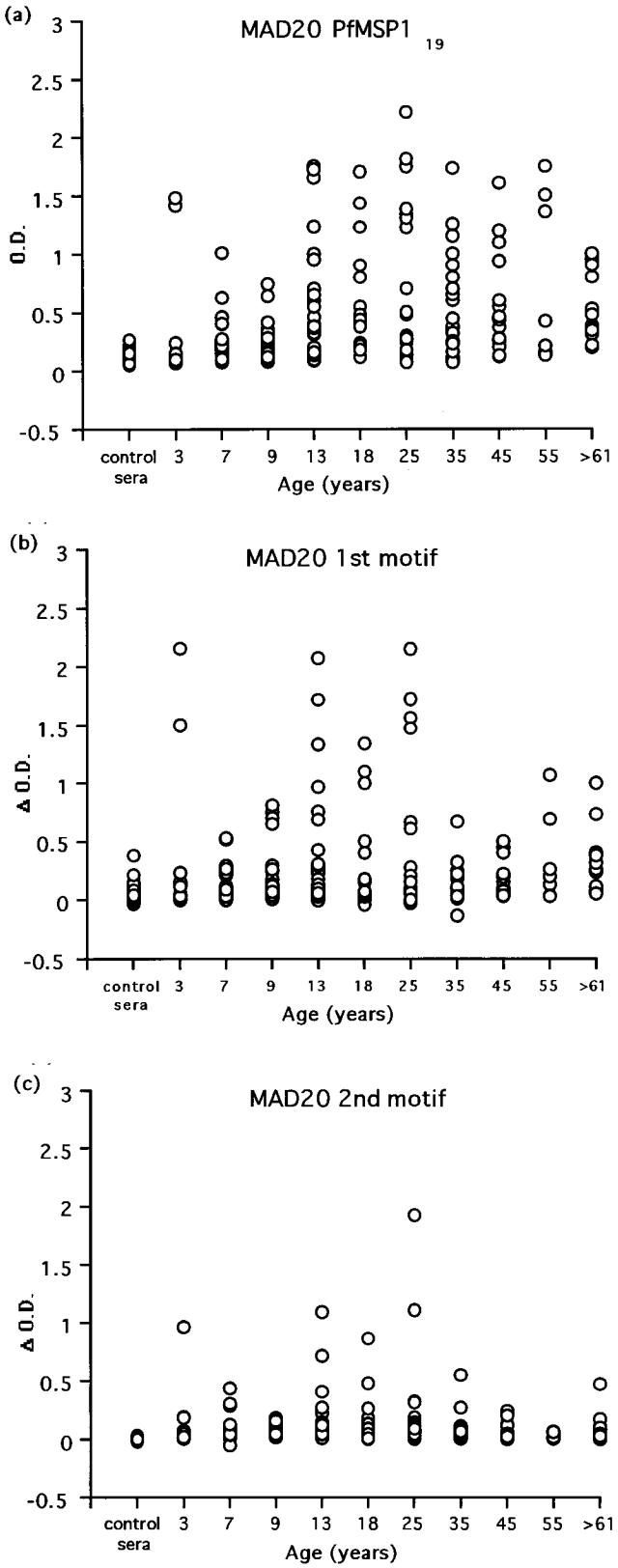


FIG. 2. Dot plots showing human antibody binding for 195 Gambian sera in an ELISA for recombinant PfMSP1 proteins. (a) MAD20 PfMSP1$_{19}$ (3D7), OD values; (b) MAD20 first motif (1B), ΔOD values (see text for details); (c) MAD20 second motif (2B), ΔOD values. The OD values of 42 European control sera are also shown. All sera were tested at a dilution of 1:3,000.

TABLE 2. Effect of age on median levels of anti-PfMSP1 antibodies[a]

| Antigen | Median OD by age group | | | Kruskal-Wallis test | |
|---|---|---|---|---|---|
| | <8 yr ($n$ = 43) | 9–15 yr ($n$ = 59) | >16 yr ($n$ = 92) | $\chi^2$ | $P$ |
| Wellcome 1st domain | 0.047 | 0.028 | 0.019 | 3.9 | 0.141 |
| MAD20 1st domain | 0.084 | 0.096 | 0.138 | 4.5 | 0.103 |
| MAD20 2nd domain | 0.042 | 0.057 | 0.058 | 1.7 | 0.428 |
| MAD20 MSP1$_{19}$ | 0.124 | 0.182 | 0.413 | 40.3 | <0.001 |
| Wellcome MSP1$_{19}$ | 0.143 | 0.215 | 0.448 | 35.5 | <0.001 |
| MAD20/Wellcome MSP1$_{19}$ | 0.120 | 0.200 | 0.411 | 36.0 | <0.001 |
| Wellcome/GST MSP1$_{19}$ | 0.024 | 0.128 | 0.307 | 23.1 | <0.001 |
| GST | 0.111 | 0.115 | 0.135 | 12.7 | 0.002 |

[a] Sera were diluted 1:3,000.

incubated with various concentrations of MAb, then with human serum, and finally with HRP-anti-human IgG.

**(iii) ELISA with reduced and nonreduced antigen.** To determine whether epitopes recognized by human antibodies were dependent on the presence of intact disulfide bonds, ELISAs were performed with reduced and alkylated or nonreduced recombinant antigens. To prepare reduced protein, 0.5 mg of an antigen was incubated at 37°C for 1 h in 0.5 M Tris-HCl (pH 8.1) containing 2 mM EDTA and 60 mM dithiothreitol (Sigma). Iodoacetic acid, dissolved in 1 M NaOH, was then added at a 2.5-fold molar excess over dithiothreitol. The samples were kept in the dark, the pH was monitored and maintained at 8.1 for 30 min, and then the proteins were dialyzed overnight at 4°C. Plates were coated for ELISA with the appropriate saturating concentration of the reduced protein and used as above.

**(iv) ELISA to determine IgG subclass.** ELISAs were performed as above (1) except that monoclonal, subclass-specific, mouse anti-human IgG was used as the second-step reagent. Subclass-specific assays were optimized by titration so that the titration curves for each reagent were coincident; i.e., that for a given absolute concentration of IgG1, IgG2, IgG3, or IgG4, the same OD value was obtained. The reagents used were mouse monoclonal anti-human IgG1 (code 1170317; Boehringer, Mannheim, Germany), IgG2 (code 1170309; Boehringer), IgG3 (code MCA516; Serotec, Oxford, United Kingdom), and IgG4 (code 1170287; Boehringer).

**Statistical methods.** Specific OD values obtained for sera from malaria-unexposed European donors were used to establish a normal range for each antigen. The OD values for these control sera tended to be normally distributed; thus, Gambian sera giving an OD value greater than the mean plus standard deviations (SDs) of the European sera were considered to contain antibody specific for the relevant recombinant protein.

The association between OD values of individual sera to the different antigens was assessed by using scatter plots and Spearman's rank correlation coefficient.

To determine whether levels of antibody to PfMSP1 proteins are associated with age, the distributions of OD values for each antigen were compared between the age groups by using the Kruskal-Wallis one-way analysis of variance. It was necessary to use a nonparametric test because the distributions of the OD values were highly skewed within each age group, and hence median OD values are presented.

## RESULTS

**Recognition of recombinant PfMSP1 proteins by human antibody.** More than 40% of sera from 195 malaria-exposed donors contain antibodies which specifically recognize the double EGF motif of PfMSP1$_{19}$ (Table 1 and Fig. 2a). In contrast, less than 20% of the sera show reactivity with the single EGF motif proteins (Fig. 2b and c [reactivity with the first EGF motif of the Wellcome version is almost identical to the data in Fig. 2b; not shown]). Of the sera which recognized MSP1$_{19}$, approximately 50% recognized only MSP1$_{19}$ and did not recognize any of the single EGF motif proteins. This indicates that there are epitopes present in PfMSP1$_{19}$ that are not present when either EGF motif is expressed alone.

Responses to the double-motif protein produced in *E. coli* (Wellcome MSP1$_{19}$ [19-GST]) and to the same sequence produced in *S. cerevisiae* (FVO) were highly correlated (Spearman's rank correlation coefficient: $r$ = 0.901, $n$ = 195, $P$ <

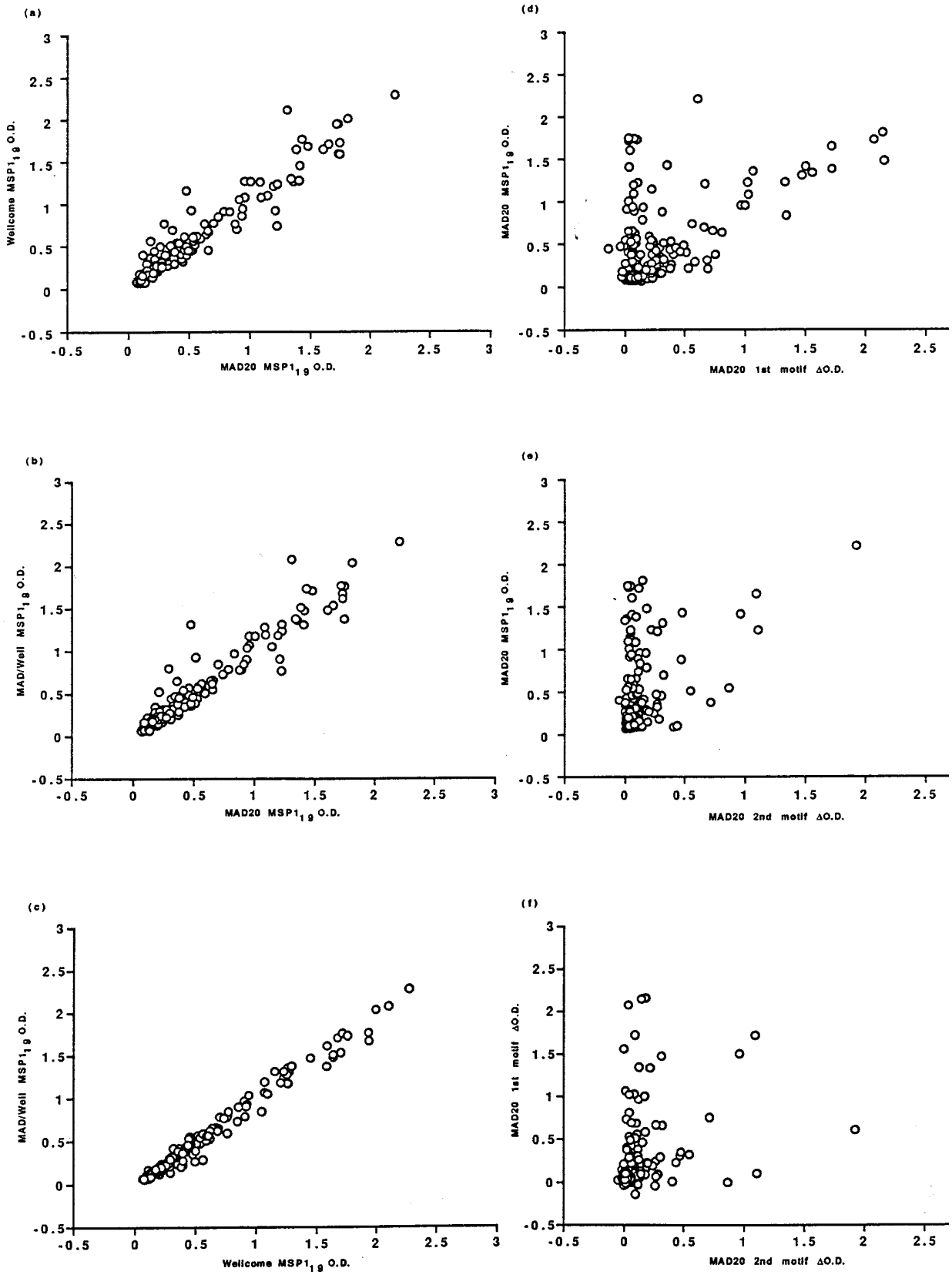


FIG. 3. Comparison of antibody recognition of different $PfMSP1_{19}$ sequences. (a) Wellcome $PfMSP1_{19}$ (FVO; Q-KNG) versus MAD20 $PfMSP1_{19}$ (3D7; E-TSR); (b) MAD20/Wellcome $PfMSP1_{19}$ (FVO/E; E-KNG) versus MAD20 $PfMSP1_{19}$ (3D7; E-TSR); (c) MAD20/Wellcome $PfMSP1_{19}$ (FVO/E; E-KNG) versus Wellcome $PfMSP1_{19}$ (FVO; Q-KNG); (d) MAD20 $PfMSP1_{19}$ (3D7) versus MAD20 first motif (1B); (e) MAD20 $MSP1_{19}$ (3D7) versus MAD20 second motif (2B); (f) MAD20 first motif (1B) versus MAD20 second motif (2B).

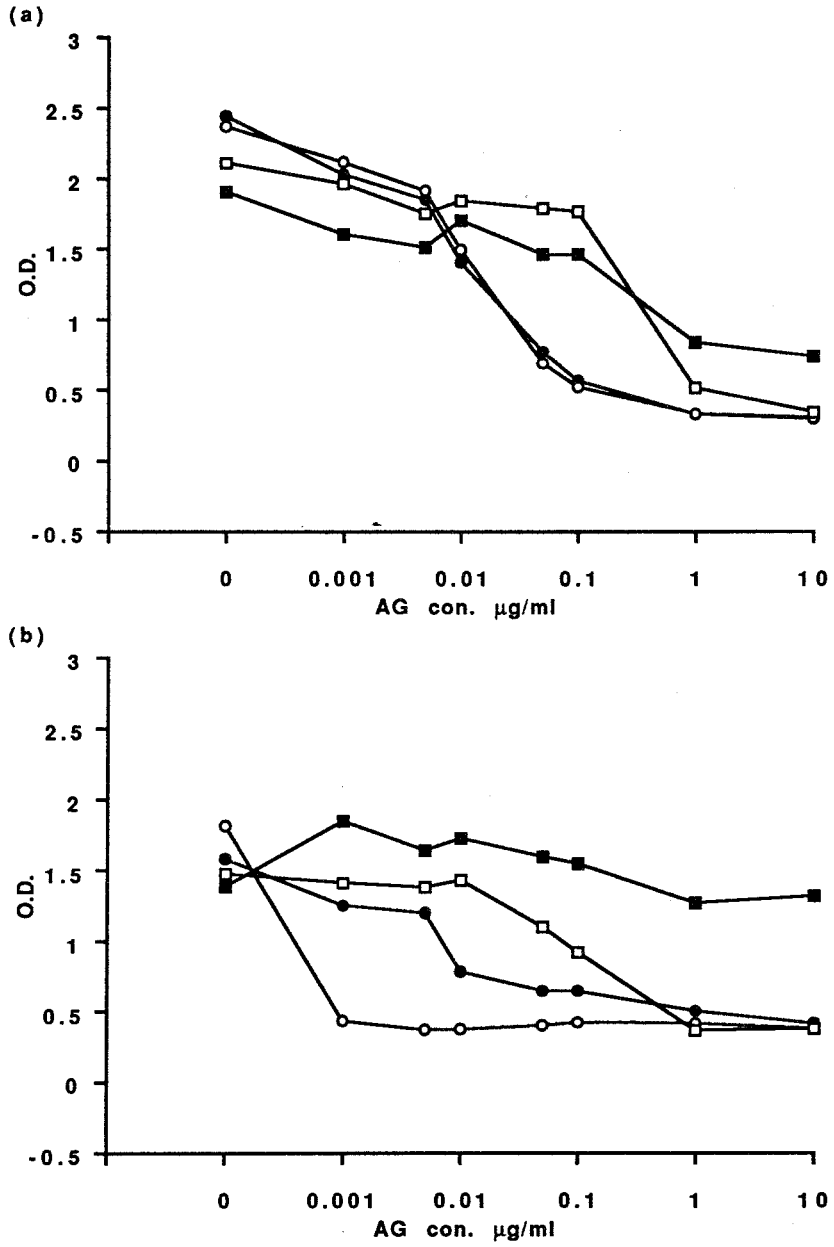


FIG. 4. Competition ELISAs demonstrate that additional epitopes are present within the double EGF motif protein (3D7). Eight sera which react with the single EGF motif (1B) and with the double EGF motif (3D7) were tested; the results for two sera are shown here. (a) Serum 1493. Preincubation of the serum with 1B reduces the level of binding to 3D7 but does not abolish it. This serum may recognize an epitope in 3D7 which overlaps with an epitope in 1B. (b) Serum 1515. Preincubation of the serum with 1B has no effect on the recognition of 3D7. This serum appears to recognize an epitope(s) in 3D7 which is distinct from the epitope(s) in 1B. ●, preincubation with 3D7, tested on 3D7; ○, preincubation with 3D7, tested on 1B; □, preincubation with 1B, tested on 1B; ■, preincubation with 1B, tested on 3D7. AG, antigen.

0.001), indicating that proteins produced in the two expression systems are very similar.

For the double-motif proteins, there is an apparent increase in antibody levels with age (Table 2); such a trend is not apparent for the single EGF motif proteins.

**Serological cross-reactivity between Wellcome and MAD20 antigens of PfMSP1$_{19}$. (i) Comparison of antibody recognition of proteins representing different PfMSP1$_{19}$ sequences.** Sera with high levels of antibody to the Wellcome proteins also tend to have high levels of antibody to the MAD20 proteins. When recognition of two double-motif proteins is directly compared, there is a high degree of correlation between them (Fig. 3a to c). However, occasional sera do react more strongly with one sequence than with the other; this seems to be due more to a differential recognition of three dimorphic amino acid residues in the second EGF motif (Fig. 3a and b) than to detection of the single-amino-acid change in the first motif (Fig. 3c). Some sera show similar levels of reactivity to both the double motif and the single first motif (Fig. 3d), whereas other sera recognize only the double motif. Similarly, occasional sera recognize both the double motif and the second single motif (Fig. 3e), and one or two sera recognize both the first and second motifs

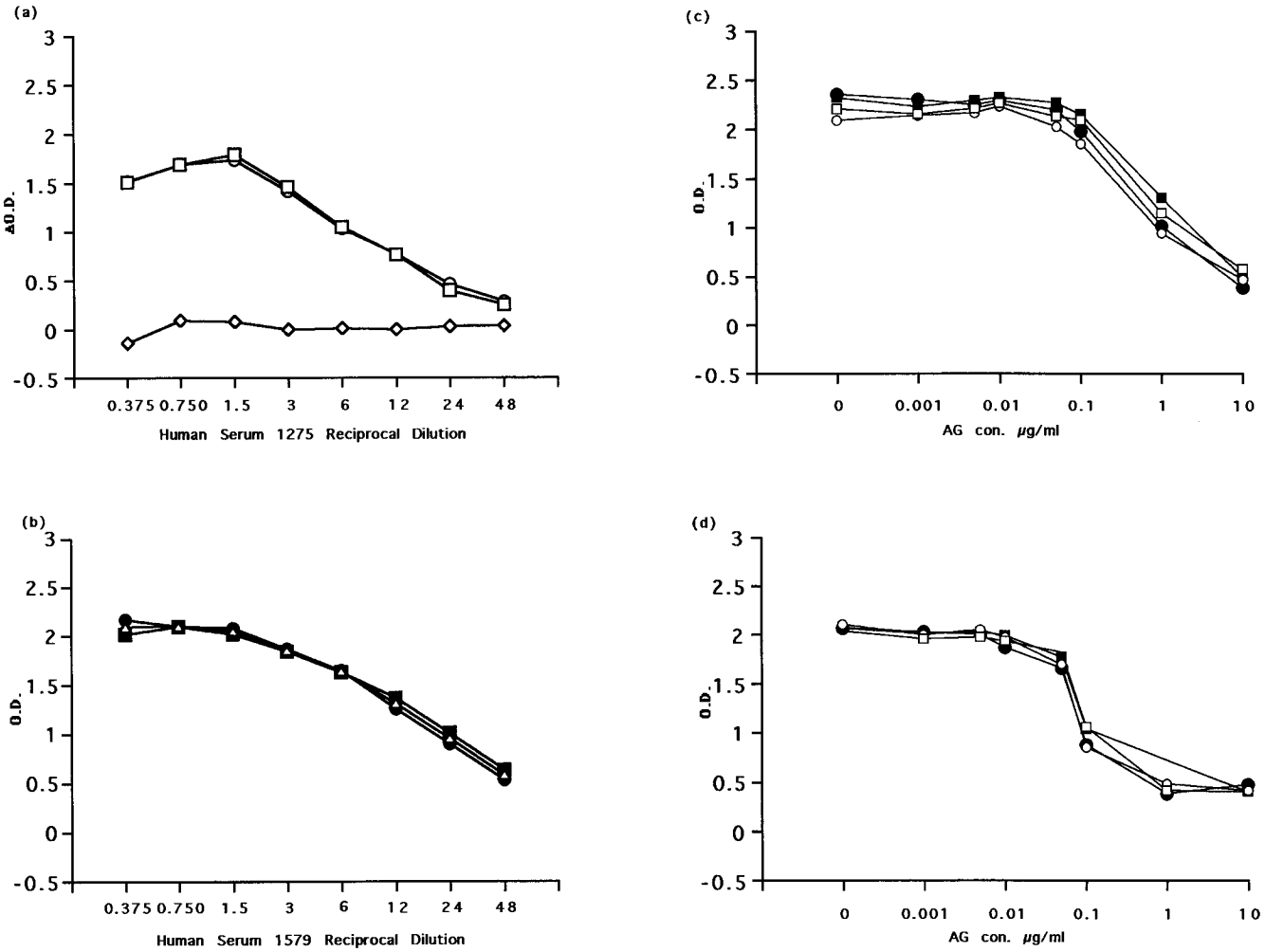


FIG. 5. Human sera recognize epitopes which are cross-reactive between MAD20 and Wellcome sequences of $PfMSP1_{19}$. (a) Human serum 1275 recognizes both sequences of the first EGF motif (1A and 1B) but not the second motif (2B). (b) Human serum 1579 recognizes all three yeast double-motif $PfMSP1_{19}$ constructs. (a and b) ○, 1A; □, 1B; ◇, 2B; ●, 3D7; △, FVO; ■, FVO/E. Dilutions are in thousands. (c and d) Preincubation of human serum 1592 (c) or 1614 (d) with 1 μg of either protein 1A or protein 1B per ml inhibits subsequent binding of that serum to plates coated with either 1A or 1B. ●, serum preincubated with 1A on 1A-coated plates; ■, serum preincubated with 1B on 1A-coated plates; ○, serum preincubated with 1A on 1B-coated plates; □, serum preincubated with 1B on 1B-coated plates.

(Fig. 3f). However, many sera (54%) which recognize the double motif do not recognize either of the single motifs. Competition ELISAs show that, of the sera which recognize both the single- and double-motif proteins, most recognize additional epitopes that are present only in the double-domain construct (two examples are shown in Fig. 4).

**(ii) Titration of individual human sera against different $PfMSP1_{19}$ proteins.** The above data suggested that most human antibodies do not differentiate between the variant sequences of $PfMSP1_{19}$. The two allelic forms of the first EGF motif differ in sequence by only one amino acid (glutamine in the Wellcome form and glutamate in the MAD20 form at position 14 [Fig. 1]); it is thus possible that the two sequences are immunologically cross-reactive. Six sera containing antibody to the first EGF motif were titrated against the three single EGF motif recombinant proteins; an example is shown in Fig. 5a. All six sera gave identical titration curves for the proteins representing the two allelic types of the first EGF motif, suggesting that a single population of antibodies recognized both proteins. All of these sera also gave identical titration curves for the three yeast-derived double-motif proteins (an example is shown in Fig. 5b).

**(iii) Polyclonal sera contain cross-reactive populations of antibodies.** To confirm that there was a single cross-reactive antibody population, competition ELISAs were performed. Individual sera which recognized MAD20 and Wellcome-derived proteins with equal avidity were selected. As shown in Fig. 5c (serum 1592), preincubation of the serum with 1 to 10 μg of either protein 1A or protein 1B per ml inhibits subsequent binding of that serum to plates coated with either 1A or 1B. For another serum (1614, Fig. 5d), inhibition was obtained by preincubation with 0.1 to 1.0 μg of protein per ml.

**Human antibodies inhibit the binding of MAbs.** $PfMSP1_{19}$-specific MAbs can block merozoite invasion of erythrocytes in vitro (1). To see if human sera recognized the same epitopes as inhibitory MAbs, competition ELISAs were performed. Five MAbs were tested: 111.4 recognizes the first motif of Wellcome specifically (1A), 12.8 and 14-1C recognize a conserved epitope in the first motif, and 12.10 and 111.2 recognize the double motif only (8; our unpublished data). Human sera with high titers of antibodies to $PfMSP1_{19}$ were able to block the binding of all these MAbs in a dose-dependent manner (examples in Fig. 6a and c), indicating that the epitopes recognized by these MAbs are also recognized by human sera. How-

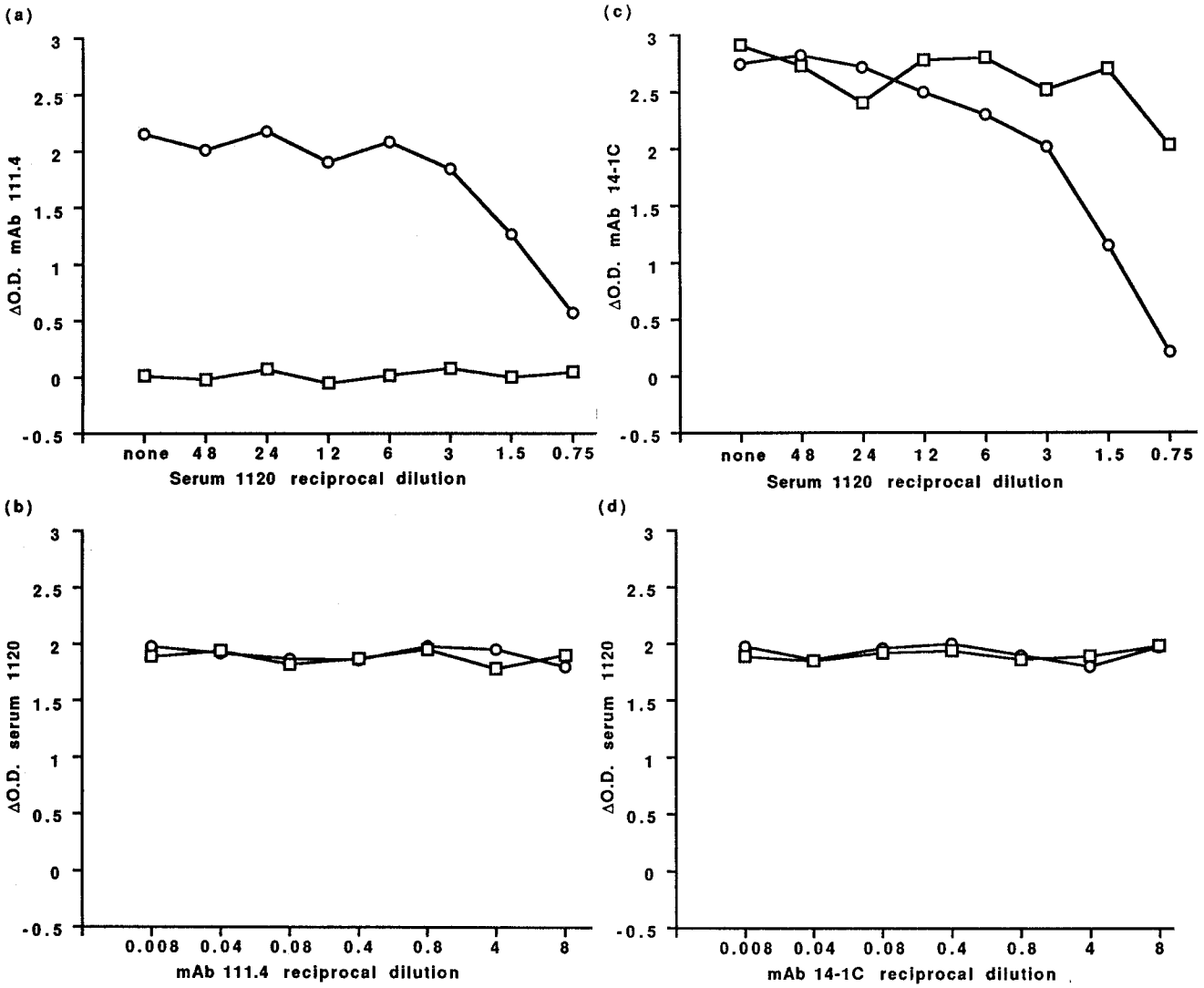


FIG. 6. Competition ELISAs. Human serum 1120 was able to inhibit the binding of MAbs in a dose-dependent manner, but MAbs were not able to inhibit binding of human antibody. (a) Serum 1120 inhibits binding of MAb 111.4 (MAb 111.4 recognizes only 1A). (b) MAb 111.4 does not inhibit binding of serum 1120. (c) Serum 1120 inhibits binding of MAb 14-1C. (d) MAb 14-1C does not inhibit binding of serum 1120. ○, 1A; □, 1B. Dilutions are in thousands.

ever, in the reciprocal experiments, MAbs were not able to completely block binding of human antibodies (Fig. 6b and d), suggesting that human sera recognize other epitopes in addition to those recognized by MAbs.

**Human antibodies recognize disulfide-dependent epitopes.** $PfMSP1_{19}$ is believed to contain three disulfide bonds in each of the two EGF motifs (3) (Fig. 1). To determine whether recognition of $PfMSP1_{19}$ by human antibodies is dependent on the presence of intact disulfide bonds, an ELISA comparing the reactivity of reduced and nonreduced forms of the recombinant antigens was performed (Table 3). We were able to show that epitopes recognized by human polyclonal antibodies are disulfide dependent, since reactivity with antigen was reduced or completely abolished after reduction and alkylation of the recombinant proteins. Recognition of the individual EGF motifs is entirely abolished after reduction, though some reactivity to the double-motif proteins remains. This suggests that minor, linear epitopes are also recognized by polyclonal human antibodies. In contrast, all the MAbs tested recognized only nonreduced antigen (Table 3).

**Predominant antibody response is IgG1.** The subclass of IgG produced in response to a given antigen determines the function of the antibody. IgG subclass was determined by subclass-specific ELISA. In most positive sera, the IgG was predominantly of the IgG1 subclass (Fig. 7). Occasional individuals produced mainly IgG3, and there was a single IgG4 positive serum for antigen 1A. The predominant IgG subclass did not vary among persons of different ages (data not shown).

## DISCUSSION

Recent studies have indicated that antibodies recognizing epitopes within the $PfMSP1_{42}$ and $PfMSP1_{19}$ processing products of PfMSP1 may be involved in protective immunity to malaria (1, 6–8, 31). The amino acid sequence of the 19-kDa fragment of PfMSP1 is relatively highly conserved between parasite isolates (22), and thus $PfMSP1_{19}$ is an attractive vaccine candidate. However, murine MAbs appear to recognize this protein in a sequence-specific manner (8, 11).

We have screened sera from a population from the Gambia

TABLE 3. Comparison of OD values for ELISA with nonreduced and reduced recombinant antigens[a]

| Antigen | OD | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MAbs | | | | | | Human sera | | | | | |
| | 14-1C | 7.5 | 111.4 | 6E2/53 | 12.10 | 4H4/34 | 1559 | 1572 | 1579 | 1584 | 1592 | 1599 |
| Wellcome 1st motif | | | | | | | | | | | | |
| Nonreduced | 2.531 | 2.688 | 2.658 | 0.170 | 0.150 | 0.193 | 1.433 | 0.129 | 0.153 | 0.145 | 1.856 | 1.188 |
| Reduced | 0.117 | 0.199 | 0.172 | 0.102 | 0.137 | 0.113 | 0.089 | 0.127 | 0.112 | 0.121 | 0.151 | 0.403 |
| MAD20 1st motif | | | | | | | | | | | | |
| Nonreduced | 2.475 | 0.479 | 0.137 | 0.181 | 0.199 | 0.130 | 1.859 | 0.184 | 0.100 | 0.126 | 1.878 | 1.168 |
| Reduced | 0.124 | 0.118 | 0.106 | 0.114 | 0.135 | 0.109 | 0.095 | 0.125 | 0.113 | 0.113 | 0.160 | 0.353 |
| MAD20 2nd motif | | | | | | | | | | | | |
| Nonreduced | 0.311 | 0.123 | 0.148 | 1.496 | 0.309 | 0.133 | 1.491 | 0.329 | 0.194 | 1.405 | 0.163 | 2.392 |
| Reduced | 0.159 | 0.138 | 0.113 | 0.191 | 0.135 | 0.125 | 0.198 | 0.251 | 0.284 | 0.355 | 0.108 | 0.410 |
| MAD20 $MSP1_{19}$ | | | | | | | | | | | | |
| Nonreduced | 1.914 | 0.995 | 0.142 | 2.130 | 0.710 | 1.721 | 1.899 | 0.824 | 2.516 | 1.270 | 1.655 | 2.623 |
| Reduced | 0.157 | 0.114 | 0.086 | 0.099 | 0.104 | 0.093 | 0.295 | 0.220 | 0.282 | 0.222 | 0.612 | 0.813 |
| MAD20/Wellcome $MSP1_{19}$ | | | | | | | | | | | | |
| Nonreduced | 1.932 | 0.241 | 0.152 | 0.140 | 0.309 | 0.104 | 1.828 | 0.691 | 2.333 | 0.829 | 1.521 | 2.334 |
| Reduced | 0.287 | 0.106 | 0.089 | 0.092 | 0.104 | 0.108 | 0.664 | 0.316 | 0.851 | 0.132 | 1.016 | 1.486 |
| Wellcome $MSP1_{19}$ | | | | | | | | | | | | |
| Nonreduced | 1.998 | 1.971 | 2.124 | 0.126 | 1.712 | 0.105 | 1.712 | 0.627 | 2.408 | 0.820 | 1.410 | 2.412 |
| Reduced | 0.211 | 0.158 | 0.249 | 0.094 | 0.132 | 0.087 | 0.523 | 0.272 | 0.655 | 0.121 | 0.824 | 1.314 |
| Wellcome/GST $MSP1_{19}$ | | | | | | | | | | | | |
| Nonreduced | 2.549 | 2.403 | 2.449 | 0.125 | 2.324 | 0.225 | 2.176 | 0.646 | 2.553 | 1.159 | 1.747 | 2.480 |
| Reduced | 0.138 | 0.138 | 0.188 | 0.091 | 0.178 | 0.080 | 0.686 | 0.272 | 0.593 | 0.143 | 0.660 | 0.905 |
| GST | | | | | | | | | | | | |
| Nonreduced | 0.262 | 0.119 | 0.208 | 0.235 | 0.234 | 0.169 | 0.105 | 0.101 | 0.108 | 0.102 | 0.121 | 0.120 |
| Reduced | 0.134 | 0.124 | 0.101 | 0.122 | 0.128 | 0.116 | 0.078 | 0.084 | 0.087 | 0.078 | 0.105 | 0.120 |

[a] Underlining is used to highlight values for positive antigen-antibody combinations. Human sera were tested at a dilution of 1:3,000, which represents the midpoint of the titration curve. MAbs were tested at a dilution of 1:1,000, which represents a saturating concentration of antibody.

with documented previous exposure to malaria for antibodies recognizing recombinant proteins representing the two allelic forms of the single and double EGF motifs of $PfMSP1_{19}$. We find that recombinant $PfMSP1_{19}$ proteins produced either in *E. coli* or in *S. cerevisiae* are equally suitable for ELISAs. Direct comparison of antibody binding to proteins produced in the two expression systems showed that they were essentially identical. The secondary structure of $PfMSP1_{19}$, which is known to be necessary for optimal recognition by MAbs raised against the native protein, appears to be appropriately formed in recombinant proteins produced in both *S. cerevisiae* and *E. coli*. Antibodies to $PfMSP1_{19}$ in immune human sera tend to recognize disulfide-dependent epitopes; however, there is some serum reactivity with reduced double-motif proteins, suggesting that conformation-independent antibodies are also present.

The prevalence of antibodies to the double EGF motif increases with age, and serum from up to 60% of adult donors aged 16 years and above contained antibodies which recognized these constructs (Table 1). However, only a minority of individuals (either children or adults) had antibodies recognizing either of the two EGF motifs when these were expressed singly. This indicates that a dominant epitope(s) requires the presence of both EGF motifs and is missing when either motif is expressed on its own.

$PfMSP1_{19}$ antibodies in human sera tend to recognize epitopes which are conserved or cross-reactive between variant sequences. Although occasional sera were shown to contain two separate, non-cross-reacting populations of antibodies, the majority of positive sera clearly recognize an epitope which is conserved or cross-reactive between the two sequences. A few sera contain antibodies which appear to bind with higher avidity to one sequence of the protein than to the other sequence. This was particularly apparent when antigens which differed in the sequence of the second EGF motif (where there are three amino acid differences) were compared, suggesting that there is a minor epitope in the second EGF motif which involves the dimorphic residues. In contrast, substitution of glutamate for glutamine in the first motif has very little effect on recognition by human antibodies. In this respect, human antibodies differ substantially from murine MAbs (such as 111.4), which recognize epitopes involving dimorphic amino acid residues. This cross-reactivity bodes well for the development of a malaria vaccine which could provide protection against all variants of the parasite.

Human sera can inhibit the binding of MAbs whose epitopes map to either EGF motif and MAbs which recognize the double-motif structure. However, none of the MAbs could inhibit binding of human antibodies. Thus, human sera may recognize a number of epitopes within $PfMSP1_{19}$, some of which are also recognized by MAbs. Alternatively, binding of human Ig to other epitopes may sterically interfere with MAb binding.

The reason why 40% of adult donors from our cross-sectional study did not possess antibodies to $PfMSP1_{19}$ is not known. All donors are known to have been exposed to malaria over many years and had high titers of antibody to malaria schizonts. Preliminary analysis of the data with respect to the known human leukocyte antigen (HLA) class II genotype of the donors (12a) does not indicate that the response to $PfMSP1_{19}$ is genetically restricted. It is possible that the seronegative donors may have antibody to other regions of PfMSP1 which inhibit the formation of antibodies to $PfMSP1_{19}$. Steric interference has been shown between MAbs binding to apparently distant PfMSP1 epitopes (35), suggesting that the N-terminal region of PfMSP1 may physically obscure $PfMSP1_{19}$ in the native protein. Studies are in progress to determine

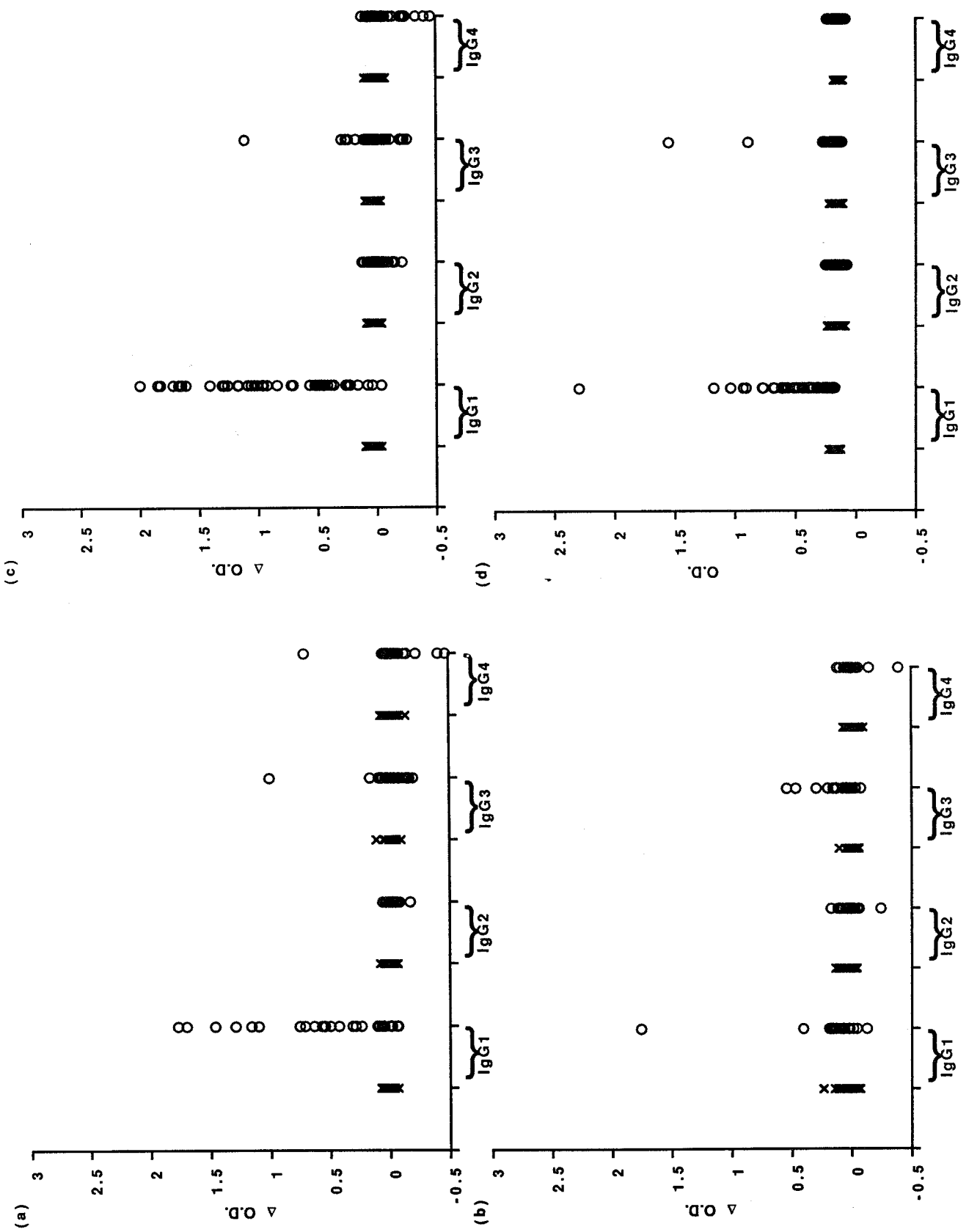
(a)
(b)
(c)
(d)
Δ O.D.
O.D.
3
2.5
2
1.5
1
0.5
0
-0.5
IgG1
IgG2
IgG3
IgG4

FIG. 7. Dot plots showing IgG subclass-specific responses. Only IgG-positive Gambian sera were tested; 24 European sera are shown as controls. All sera were tested at a dilution of 1:1,000. (a) Wellcome first motif (1A); 32 Gambian sera tested. (b) MAD20 second motif (2B); 24 Gambian sera tested. (c) Wellcome $PfMSP1_{19}$ (19/GST); 52 Gambian sera tested. (d) MAD20 $PfMSP1_{19}$ (3D7); 54 Gambian sera tested. ×, European sera; ○, Gambian sera.

patterns of antibody recognition of other regions of PfMSP1 by these sera. Alternatively, seronegative individuals may not have been recently infected with malaria, and in the absence of boosting, antibody may have fallen below detectable levels.

An important outstanding question is whether antibodies to $PfMSP1_{19}$ are protective or not. The fact that PfMSP1 appears to induce predominant IgG1 antibodies suggests that such antibodies may play a role in opsonization or complement-mediated lysis of free merozoites. Recent experiments have shown that the membrane-bound 15-kDa fragment of MSP1 from *P. yoelii* (PyMSP15) is the target of a protective MAb (6), and vaccination of mice with a recombinant protein representing the double EGF motifs of $PyMSP1_{15}$ protects against challenge infection with the 17XL and YM strains of *P. yoelii* (12, 25). A longitudinal immunoepidemological study suggested that antibodies to $PfMSP1_{42}$ were associated with resistance to clinical malaria and high parasitaemia in young children (31). We have recently completed a similar longitudinal study of antimalarial antibody levels and malaria morbidity, which indicates that antibodies to $PfMSP1_{19}$ are associated with protection from clinical malaria (unpublished data). Further studies are in progress in our laboratories to determine the functional role of human antibodies to $PfMSP1_{19}$ and their role in protective immunity to malaria.

## ACKNOWLEDGMENTS

We thank B. M. Greenwood for permitting access to serum samples collected in the Gambia. We thank A. Saul and R. Reese for gifts of MAbs.

This work was funded by grants from the Wellcome Trust and the UK Medical Research Council. J.M. is a Wellcome Senior Lecturer, and E.M.R. is a Wellcome Senior Research Fellow.

## REFERENCES

1. **Blackman, M. J., H.-G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red blood cell invasion and is the target of invasion-inhibiting antibodies. J. Immunol. **172:**379–382.
2. **Blackman, M. J., and A. A. Holder.** 1992. Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of $MSP1_{33}$ as a non-covalently associated complex with other fragments of the MSP1. Mol. Biochem. Parasitol. **50:**307–316.
3. **Blackman, M. J., I. T. Ling, S. C. Nicholls, and A. A. Holder.** 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. Mol. Biochem. Parasitol. **49:**29–34.
4. **Blackman, M. J., H. Whittle, and A. A. Holder.** 1991. Processing of the *Plasmodium falciparum* merozoite surface protein-1: identification of a secondary processing product which is shed prior to erythrocyte invasion. Mol. Biochem. Parasitol. **49:**35–44.
5. **Burghaus, P. A., and A. A. Holder.** 1994. Expression of the 19-kilodalton carboxy-terminal fragment of the *Plasmodium falciparum* merozoite surface protein-1 in *Escherichia coli* as a correctly folded protein. Mol. Biochem. Parasitol. **64:**165–169.
6. **Burns, J. M., L. A. Parke, T. M. Daley, L. A. Cavacini, W. P. Weidanz, and C. A. Long.** 1989. A protective monoclonal antibody recognises a variant-specific epitope in the precursor of the major merozoite surface antigen of the rodent parasite *Plasmodium yoelii*. J. Immunol. **142:**2835–2840.
7. **Chang, S. P., H. L. Gibson, C. T. Lee-Ng, P. J. Barr, and G. S. N. Hui.** 1992. A carboxyl-terminal fragment of *Plasmodium falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth. J. Immunol. **149:**548–555.
8. **Chappel, J. A., and A. A. Holder.** 1993. Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion in vitro recognise the first growth factor-like domain of merozoite surface protein-1. Mol. Biochem. Parasitol. **60:**303–312.
9. **Cheung, A., J. Leban, A. R. Shaw, B. Merkli, J. Stocker, C. Chizzolini, C. Sander, and L. H. Perrin.** 1986. Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. Proc. Natl. Acad. Sci. USA **83:**8328–8332.
10. **Cooke, R. M., A. J. Wilkinson, M. Baron, A. Pastore, M. J. Tapin, I. D. Campbell, H. Gregory, and B. Sheard.** 1987. The solution structure of human epidermal growth factor. Nature (London) **327:**339–341.
11. **Cooper, J. A., L. T. Cooper, and A. J. Saul.** 1992. Mapping of the region predominantly recognised by antibodies to *Plasmodium falciparum* merozoite surface antigen MSA 1. Mol. Biochem. Parasitol. **51:**301–312.
12. **Daly, T. M., and C. A. Long.** 1993. A recombinant 15-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii yoelii* 17XL merozoite surface protein-1 induces a protective immune response in mice. Infect. Immun. **61:** 2462–2467.

12a. **Egan, A., and E. Riley.** Unpublished data.

13. **Etlinger, H. M., P. Caspers, H. Matile, H.-J. Schoenfeld, D. Stueber, and B. Takacs.** 1991. Ability of recombinant or native proteins to protect monkeys against heterologous challenge with *Plasmodium falciparum*. Infect. Immun. **59:**3498–3503.
14. **Freeman, R. R., and A. A. Holder.** 1983. Surface antigens of malaria merozoites: a high molecular weight precursor is processed to an 83,000 mol wt form expressed on the surface of *Plasmodium falciparum* merozoite. J. Exp. Med. **158:**1647–1653.
15. **Greenwood, B. M., A. K. Bradley, A. M. Greenwood, P. Byass, K. Jammeh, K. Marsh, S. Tulloch, F. S. J. Oldfield, and R. Hayes.** 1987. Mortality and morbidity from malaria among children in a rural area of the Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg. **87:**478–486.
16. **Hall, R., J. E. Hyde, M. Goman, D. L. Simmons, I. A. Hope, M. Mackay, J. Scaife, R. Richle, and J. Stocker.** 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature (London) **311:**379–382.
17. **Herrera, S., M. A. Herrera, B. L. Perlaza, Y. Burki, P. Caspers, H. Dobeli, D. Rotmann, and U. Certa.** 1990. Immunization of *Aotus* monkeys with *Plasmodium falciparum* blood-stage recombinant proteins. Proc. Natl. Acad. Sci. USA **87:**4017–4021.
18. **Holder, A. A., and R. R. Freeman.** 1984. The three major antigens on the surface of *P. falciparum* merozoites are derived from a single high molecular weight precursor. J. Exp. Med. **160:**624–629.
19. **Holder, A. A., R. R. Freeman, and C. I. Newbold.** 1983. Serological cross-reaction between high molecular weight proteins synthesized in blood schizonts of *Plasmodium yoelii*, *Plasmodium chabaudi* and *Plasmodium falciparum*. Mol. Biochem. Parasitol. **9:**191–196.
20. **Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, V. Riveros-Moreno, S. C. Nicholls, Y. Hillman, L. S. Davey, M. L. V. Schwarz, and R. R. Freeman.** 1985. Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. Nature (London) **317:**270–273.
21. **Howard, R. F., and R. T. Reese.** 1984. Synthesis of merozoite proteins and glycoproteins during the schizogony of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **10:**319–34.
22. **Jongwutiwes, S., K. Tanabe, and H. Kanbara.** 1993. Sequence conservation in the C-terminal part of the precursor to the major merozoite surface proteins (MSP1) of *Plasmodium falciparum* from field isolates. Mol. Biochem. Parasitol. **59:**95–100.
23. **Kaslow, D. C., G. Hui, and S. Kumar.** 1994. Expression and antigenicity of *Plasmodium falciparum* major merozoite surface protein ($MSP1_{19}$) variants secreted from *Saccharomyces cerevisiae*. Mol. Biochem. Parasitol. **63:**283–289.
24. **Kemeny, D. M.** 1992. Titration of antibodies. J. Immunol. Methods **150:**57–76.
25. **Ling, I. T., S. A. Ogun, and A. A. Holder.** 1994. Immunisation against malaria with a recombinant protein. Parasite Immunol. **16:**63–67.
26. **McBride, J. S., and H.-G. Heidrich.** 1987. Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. Mol. Biochem. Parasitol. **23:**71–84.
27. **Miller, L. H., T. Roberts, M. Shahabuddin, and T. F. McCutchan.** 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol. Biochem. Parasitol. **59:**1–14.
28. **Patarroyo, M. E., P. Romero, M. L. Torres, P. Clavijo, A. Moreno, A. Martinez, R. Rodriguez, F. Guzman, and E. Cabezas.** 1987. Induction of protective immunity against experimental infection with malaria using synthetic peptides. Nature (London) **328:**629–632.
29. **Perrin, L. H., B. Merkli, M. Loche, C. Chizzolini, J. Smart, and R. Richle.** 1984. Antimalarial immunity in *Saimiri* monkeys: immunization with surface components of asexual blood stages. J. Exp. Med. **160:**441–451.

30. **Pirson, P. J., and M. E. Perkins.** 1985. Characterisation with monoclonal antibodies of a surface antigen of *Plasmodium falciparum* merozoites. J. Immunol. **134:**1946–1951.
31. **Riley, E. M., S. J. Allen, J. G. Wheeler, M. J. Blackman, S. Bennett, B. Takacs, H. J. Schonfeld, A. A. Holder, and B. M. Greenwood.** 1992. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. Parasite Immunol. **14:**321–337.
32. **Schmidt-Ullrich, R., J. Brown, H. Whittle, and P. S. Lin.** 1986. Human-human hybridomas secreting monoclonal antibodies to the Mr 195,000 *Plasmodium falciparum* blood stage antigen. J. Exp. Med. **163:**179–188.
33. **Siddiqui, W. A., L. Q. Tam, K. J. Kramer, G. S. N. Hui, S. E. Case, K. M. Yamaga, S. P. Chang, E. B. T. Chan, and S.-C. Kan.** 1987. Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA **84:**3014–3018.
34. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
35. **Wilson, C. F., R. Anand, J. T. Clark, and J. S. McBride.** 1987. Topography of epitopes on a polymorphic schizont antigen of *Plasmodium falciparum* determined by the binding of monoclonal antibodies in a two-site radioimmunoassay. Parasite Immunol. **9:**737–746.