Characterization of Human T- and B-Cell Epitopes in the C Terminus of *Plasmodium falciparum* Merozoite Surface Protein 1: Evidence for Poor T-Cell Recognition of Polypeptides with Numerous Disulfide Bonds

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We have investigated the relationship between cellular and humoral immune responses to defined epitopes of the C terminus of merozoite surface protein 1 (MSP-1) of the human malaria parasite, *Plasmodium falciparum*, in immune blood donors. Sera from almost all donors contained antibodies to the 33-kDa processing product of the MAD20 allele of MSP-1 (MSP-1₃₃), but these antibodies did not cross-react with the equivalent sequence of the Wellcome allele. In contrast, T-cell responses to MSP-1₃₃ are directed towards epitopes that are conserved between the two allelic families. Only 50% of adult blood donors possessed antibodies which recognized the 19-kDa processing product of MSP-1 (MSP-1₁₉). These antibodies predominantly recognized conserved epitopes involving both of the constituent epidermal growth factor-like domains of MSP-1₁₉. T-cell responses were found in only 26% (for recombinant proteins) or 44% (for synthetic peptides) of donors and were directed mainly at dimorphic sequences of the protein. There was no obvious association, at an individual level, between the presence of antibodies and the detection of T-cell proliferative or gamma interferon responses, suggesting that the T cells identified in this manner are not providing significant levels of help to B cells. T-cell responses to reduced recombinant proteins and linear peptides were more prevalent than responses to disulfide-bonded proteins, suggesting that the complex disulfide-bonded structure of native MSP-1₁₉ may inhibit antigen processing or presentation.

In the search for a subunit vaccine to confer protective immunity to *Plasmodium falciparum* malaria, much recent attention has focused on the major merozoite surface protein, MSP-1. The protein is naturally immunogenic, and vaccination experiments have shown that immunization of nonhuman primates with native or recombinant MSP-1 confers varying degrees of resistance to challenge infection with *P. falciparum* (7, 15, 18, 20, 25, 33). The C terminus of the molecule contains two epidermal growth factor (EGF)-like domains (5), and monoclonal antibodies to this region of MSP-1 inhibit erythrocyte invasion in vitro (3, 8). Immunization of mice with the equivalent region of MSP-1 from the rodent malaria agent *Plasmodium yoelii* confers almost complete resistance to lethal challenge infection (12, 26). Seroepidemiological studies have demonstrated significant associations between the presence of antibodies to the C-terminal region of the molecule and resistance to clinical malaria (1, 14, 31), and affinity-purified human antibodies to the C terminus of MSP-1 inhibit parasite growth in vitro (12b).

Posttranslational processing of MSP-1 produces a non-covalently linked complex, of which only the C-terminal 42-kDa fragment (MSP-1₄₂) is directly attached to the merozoite surface via a glycosylphosphatidylinositol anchor (19) (see Fig. 1a). Secondary processing of MSP-1₄₂ produces a membrane-

bound 19-kDa polypeptide (MSP-1₁₉) composed of the two EGF-like domains (3, 5) and a 33-kDa polypeptide (MSP-1₃₃) which is shed from the merozoite surface prior to erythrocyte invasion (4).

Multiple alleles of the MSP-1 gene, encoding proteins of differing sizes and sequences, have been identified (27, 35) and can be classified into two allelic families, the Wellcome/K1 family and the MAD20 family. MSP-1₃₃ is essentially dimorphic, with considerable sequence diversity between the two allelic families, but MSP-1₁₉ is highly conserved, with only four amino acid differences in 96 residues (27). The N terminus of the molecule is more polymorphic, with three major families and multiple sequence variants (23, 29).

We have previously investigated the human antibody response to MSP-1₄₂, MSP-1₃₃, and MSP-1₁₉ in malaria-endemic populations and have shown that antibody responses appear to be directed mainly towards variant epitopes within MSP-1₃₃; more than 95% of malaria-exposed Gambian sera recognize MAD20 MSP-1₃₃, but few appear to recognize epitopes within Wellcome MSP-1₃₃ (12a). This correlates with the known prevalences of the two alleles in west Africa, where more than 95% of parasite isolates are of the MAD20 genotype (10). In contrast, only 50% of clinically immune adults possess antibodies which bind to MSP-1₁₉; in general, these antibodies recognize disulfide-dependent epitopes formed by the two EGF-like domains together and do not differentiate between the two variant sequences of MSP-1₁₉ (13).

The low prevalence of antibodies to MSP-1₁₉ is maintained over time (36) despite persistent reexposure to antigen, raising the possibility that lack of appropriate T-cell help may be a

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FIG. 1. Schematic diagram of *P. falciparum* MSP-1 showing the processing products, the dimorphic residues in MSP-1₁₉, and the recombinant and synthetic proteins used. (a) Primary and secondary processing of MSP-1. Numbers denote the apparent molecular masses of the fragments (in kilodaltons) as determined by SDS-PAGE. EGF, EGF-like domains. The zigzag lines at the right indicate the glycosylphosphatidylinositol anchors (the exact amino acid at the C terminus has not been determined). (b) Primary sequences of MAD20 and Wellcome alleles of MSP-1₁₉ showing the positions of synthetic peptides (■) and recombinant antigens (□). Lowercase letters denote dimorphic residues. Well-19 and MAD20-19 are as defined in the footnotes for Table 1.

contributory factor. Previous studies, by ourselves and others, with native and recombinant MSP-1 antigens have suggested that there are immunodominant T-cell epitopes in the N terminus of MSP-1 (11, 17, 31, 32, 34), but systematic analysis of the response to the C-terminal region of the molecule has been hampered by the lack of antigens representing both dimorphic sequences. In the present study we have conducted a comprehensive analysis of T-cell responses to antigens representing both sequence variants of MSP-1 and have looked for associations between seropositivity and T-cell responsiveness. In addition, we provide evidence that the structure of MSP-1₁₉, with its numerous disulfide bonds, may inhibit antigen processing or presentation and that reduction of the antigen prior to presentation to T cells can, at least in vitro, significantly enhance its recognition.

MATERIALS AND METHODS

Subjects and samples. Malaria-exposed donors were volunteers recruited from a rural village on the south bank of the Gambia River where *P. falciparum* malaria is seasonally endemic (16). Peak malaria transmission occurs from August to November, following the annual rainy season (July to October). All the donors were adult (age, >18 years), lifelong residents of the area who experience occasional asymptomatic malaria infections but who are resistant to the clinical symptoms of infection (clinically immune). All samples were collected during the dry season (season of little malaria transmission) in June (donors 1 to 16) or in April (donors 17 to 35). Venous blood samples were collected, placed into heparinized tubes, and transported in insulated boxes to the laboratory for processing (transit time, approximately 2 h). After centrifugation, plasma was removed and stored (at -20°C) for antibody estimations.

Giemsa-stained thick-blood films were examined for the presence of circulating malaria parasites. All 35 donors were parasite negative at the time of blood sampling. Six malaria-unexposed European donors provided control samples for T-cell assays, and plasma samples from 40 adult Europeans were used as serological controls.

Antigens. (i) Recombinant antigens. The individual EGF-like domains of MSP-1₁₉, the Wellcome version of the double-domain 19-kDa protein, and the MAD20 version of MSP1₃₃ were produced as fusion proteins with the glutathi-

one S-transferase (GST) of *Schistosoma japonicum* by using pGEX vectors transfected into *Escherichia coli*. GST, produced from vectors lacking an MSP-1 insert, was used as a negative control. The recombinant pGEX plasmids for proteins Well-EGF-1, MAD20 EGF-2, and the Wellcome version of MSP-1₁₉ (Fig. 1b) were kind gifts from Jon Chappel and Petra Burghaus (National Institute for Medical Research, London, United Kingdom). The production and purification of these antigens have been described in detail elsewhere (6, 8, 12a). The MAD20 version of MSP-1₁₉ was a kind gift from David Kaslow (National Institutes of Health, Bethesda, Md.) and was expressed as a soluble protein, with a hexahistidine tail, in *Saccharomyces cerevisiae*. The protein was purified on nickel-nitrilotriacetic acid agarose (24). Finally, the Wellcome MSP-1₄₂ protein was produced as a soluble baculovirus product, as described previously (28). All antigens were extensively dialyzed before use, and purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The specificity of the response was ensured by the use of a control dialysis medium and by the testing of cells from malaria-nonexposed donors. All the recombinant antigens are believed to conform closely to the secondary and tertiary structures of the native protein because they are recognized with a high degree of affinity by conformation-dependent monoclonal antibodies (6, 8) and because antiserum to recombinant proteins recognizes native protein in immunoblot or immunofluorescence testing of whole parasites (12a).

(ii) Synthetic peptides. Nine overlapping peptides (eight 25-mers and one 21-mer) (Fig. 1b), representing both variants of the entire MSP-1₁₉ sequence, were synthesised by solid-phase peptide synthesis (PACemaker; Affiniti Research Products Ltd., Nottingham, United Kingdom) and purified by high-performance liquid chromatography. Purity ranged from >80 to >95%. Peptides were reconstituted in sterile phosphate-buffered saline (PBS) and were UV irradiated to ensure sterility.

(iii) Control antigens. The mitogen phytohemagglutinin (PHA; Sigma, Poole, United Kingdom) and two recall antigens, purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Evans Medical Ltd., Leatherhead, United Kingdom) and tetanus toxoid (TT) (Wellcome Biotechnology, Beckenham, United Kingdom), were used as positive controls for testing lymphocyte viability and responsiveness.

(iv) Reduction and alkylation of antigens. Recombinant MSP-1 antigens and control antigens (PPD and TT) were reduced by incubation for 1 h at 37°C 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 added at a 2.5-fold molar excess over dithiothreitol, and the samples were kept in the dark at a constant pH of 8.1 for 30 min. Antigens were then dialyzed overnight at 4°C. The protein concentration was recalculated, and the reduced and alkylated antigens were reconstituted in sterile PBS.

TABLE 1. Responses to recombinant MSP-1 proteins for donors 1 to 16

Donor no.	T-cell proliferative response (SI) ^a to:						Antibody response (sA ₄₉₂) to:					
	WELL/ EGF-1	MAD20 EGF-1	MAD20 EGF-2	MAD20 19 ^b	WELL 19 ^c	WELL 42 ^d	WELL/ EGF-1	MAD20 EGF-2	MAD20 19	WELL 19	MAD20 33	WELL 42
1						>0.5					>0.5	
2						2-5						
3						2-5	0-0.5		0-0.5	0-0.5		0-0.5
4	2-5					2-5			0-0.5	0-0.5	>0.5	0-0.5
5						2-5	0-0.5				>0.5	
6				2-5		2-5					>0.5	
7						2-5					>0.5	
8											0-0.5	0-0.5
9						2-5					0-0.5	
10				2-5		2-5			0-0.5	0-0.5		0-0.5
11						>0.5		0-0.5		0-0.5	0-0.5	0-0.5
12						2-5					>0.5	
13				2-5		>0.5					>0.5	
14				2-5	2-5	>0.5				>0.5	>0.5	0-0.5
15						>0.5					0-0.5	
16						2-5	>0.5		>0.5	>0.5	>0.5	>0.5

^a The absence of data indicates that no response was observed. SIs were calculated relative to that of the GST control. Counts-per-minute values for GST alone ranged from 616 to 4,451 for the 16 donors.

^b MAD20 19, the MAD20 version of MSP-1₁₉.

^c WELL 19, the Wellcome version of MSP-1₁₉.

^d WELL 42, the Wellcome version of MSP-1₄₂.

T-cell proliferation assays. Mononuclear cells were separated from heparinized blood by centrifugation through Lymphoprep (sodium metrizoate/Ficoll; Pharmacia, Uppsala, Sweden), washed in RPMI 1640 (Gibco, Paisley, United Kingdom), and resuspended at a concentration of 10⁶ viable cells per ml in complete culture medium (RPMI 1640 with 2 mM L-glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, 30 mM HEPES, 0.22% [vol/vol] sodium bicarbonate; Sigma) with 10% heat-inactivated, nonimmune human serum. Cells were aliquoted (100 µl/well) into sterile, round-bottomed, 96-well microtiter plates. Antigens, in 100 µl of complete medium, were added to triplicate wells, and plates were incubated at 37°C in 5% CO₂ for 7 days. Eighteen hours before harvesting, 100 µl of supernatant was removed from each well and reserved for cytokine analysis. The supernatant was replaced with 100 µl of fresh medium containing 1 µCi of [³H]thymidine (Amersham Life Sciences, Little Chalfont, United Kingdom). Cells were harvested onto cellulose filters, and incorporation of radiolabelled thymidine was assessed by liquid scintillation counting.

The geometric mean (gm) counts per minute was determined for each antigen, and the stimulation index (SI) was calculated as the ratio of antigen-stimulated to control counts per minute. In the case of GST fusion proteins, the counts-per-minute value for GST-stimulated cells was used as the control.

IFN-γ assay. Six-day cell culture supernatants were tested for the presence of gamma interferon (IFN-γ) by immunoassay (2) using a two-site capture enzyme-linked immunosorbent assay (ELISA; Chromogenix, Mölndal, Sweden). IFN-γ concentration was determined by reference to the international human IFN-γ standard, GG23-901-530 (National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Antibody assays. Antibody levels were determined by indirect ELISA (13). Briefly, flat-bottomed microtiter plates (Dynatech, Billingshurst, United Kingdom) were coated overnight with optimal concentrations of antigen (varying between 0.5 and 5.0 µg/ml) in carbonate-bicarbonate coating buffer. Washed plates were blocked with a 1% solution of nonfat milk powder in PBS/T (PBS with 0.05% Tween 20; Sigma), and plasma (diluted 1:1,000 in blocking buffer) was added to duplicate wells. Plates were incubated for 1 h at 37°C, washed three times with PBS/T, and developed with rabbit anti-human immunoglobulin G (Dako Ltd, High Wycombe, United Kingdom), followed by hydrogen peroxide plus o-phenylenediamine (Sigma). The reaction was stopped with 20 µl of 2 M H₂SO₄ per well, and absorbance was read at 492 nm (A₄₉₂).

For GST fusion proteins, the absorbance value for the GST control was subtracted from the absorbance for the fusion protein to give a specific absorbance (sA₄₉₂) for the response to the malaria antigen.

Plasma samples from 40 malaria-unexposed adults were used as negative controls. The mean plus 2 standard deviations for the sA₄₉₂ of the controls was taken as the upper limit of the normal range. Test samples giving sA₄₉₂ values above this value were regarded as positive. Samples giving sAbs values of >0.5 unit above the cutoff were regarded as highly positive.

RESULTS

Two separate sets of experiments were performed. In the first, 16 adult donors were sampled and T-cell responses to both recombinant antigens and synthetic peptides were tested. In the second, 19 donors were sampled and T-cell responses to native and reduced recombinant antigens were tested. Plasma samples from all 35 donors were tested for antibodies to both recombinant antigens and synthetic peptides.

T-cell proliferation. Positive proliferative responses were defined as SIs greater than 2.0. The cutoff was set deliberately low to ensure that no positive responses were missed and to ensure that the prevalence of responses was not underestimated. All 35 donors made strong proliferative responses to PHA (SI range, 9.2 to 552.1), indicating that the cells were viable. In addition, 34 of the 35 donors made a response to *M. tuberculosis* PPD (SI range, 1.4 to 475.8) and 23 of the 35 made a response to TT (SI range, 0.7 to 159.3).

(i) Recognition of recombinant antigens. The control donors responded to recombinant MSP-1 antigens with SIs of between 0.3 and 2.3 when antigens were used at a final concentration of 1 or 5 µg/ml and with SIs of 0.5 to 4.5 at antigen concentrations of 10 µg/ml. Responses above SI = 2.5 in control donors were considered to be nonspecific (i.e., possibly induced by cross-reacting antigens), and we thus chose to use antigens at concentrations which did not induce such nonspecific responses. Cells from each malaria-exposed donor were therefore tested at two antigen concentrations (1 and 5 µg/ml), and the higher SI (which was frequently at the 1-µg/ml concentration) was taken to be the optimum response. The cutoff for positive responses was taken as SI of >2.0.

Fifteen of the 16 donors tested (94%) responded to MSP-1₄₂ with SIs greater than 2.0; for 5 of these 15 responders, the SIs were greater than 5 (Table 1). In contrast, cells from only 9 of 35 donors (26%) proliferated in response to any of the recombinant MSP-1₁₉ proteins (Tables 1 and 2). Of these nine donors, cells from only two donors (donors 14 and 25) responded to proteins representing both allelic sequences of MSP-1₁₉.

TABLE 2. Responses to recombinant MSP-1 proteins for donors 17 to 35

Donor no.	T-cell proliferative response (SI ^a) to:											Antibody response (aA ₄₉₂) to:						
	Nonreduced antigen:						Reduced antigen:											
	WELL/ EGF-1	MAD20 EGF-1	MAD20 EGF-2	WELL 19	MAD20 19	TT	WELL/ EGF-1	MAD20 EGF-1	MAD20 EGF-2	WELL 19	MAD20 19	TT	MAD20 EGF-1	MAD20 EGF-2	WELL 19	MAD20 19	MAD20 33	WELL 42
17										2-5	>5							>0.5
18											>5							>0.5
19						>5		2-5			>5	0-0.5		>0.5	>0.5		>0.5	>0.5
20	2-5					>5	>5	>5	>5		2-5						>0.5	
21						2-5				2-5	>5			>0.5	>0.5		>0.5	>0.5
22	2-5			2-5					2-5						>0.5	>0.5		>0.5
23							2-5	>5	>5	2-5							0-0.5	
24														>0.5	0-0.5		>0.5	>0.5
25	2-5	2-5					2-5	>5	2-5	>5	2-5						>0.5	
26							2-5	2-5	2-5		2-5	>5	0-0.5			0-0.5	0-0.5	0-0.5
27						>5					>5	2-5					>0.5	
28			2-5								>5	2-5					>0.5	
29						>5		>5		2-5	2-5			>0.5	0-0.5		>0.5	0-0.5
30											2-5	2-5	>0.5				0-0.5	
31						>5					>5	>5	>0.5				>0.5	0-0.5
32														0-0.5	0-0.5	>0.5	ND ^b	>0.5
33							2-5	2-5	2-5	2-5	2-5	2-5					>5	
34						>5					2-5	2-5	0-0.5		0-0.5	0-0.5	>0.5	0-0.5
35						>5					2-5	>5					0-0.5	

^a The absence of data indicates that no response was observed. SIs were calculated relative to that of the GST control. Counts-per-minute values for GST alone ranged from 127 to 2,399 for nonreduced GST and from 116 to 5,084 for reduced GST. The protein designations are as defined in the footnotes to Table 1.

^b ND, not done.

These data suggest that T-cell epitopes in MSP-1₃₃ are commonly recognized but that epitopes in MSP-1₁₉ are infrequently recognized. (It was not possible to test T-cell responses to the MSP-1₃₃ antigen directly because the recombinant protein was toxic to cells.) In addition, epitopes in the 19-kDa region tend to span the dimorphic residues such that T-cell recognition is allele specific. The fact that almost all individuals had T cells which recognized the Wellcome allelic sequence of MSP-1₄₂, despite the low level of exposure to this allele in The Gambia, suggests that the T-cell epitopes in MSP-1₃₃ are in conserved parts of the sequence.

(ii) **Recognition of synthetic peptides.** Peptides were tested for potential cytotoxicity by adding peptide, at concentrations of 1 or 10 µg/ml, to cultures of cells from European donors containing optimum concentrations of either *M. tuberculosis* PPD or PHA. The responses to PHA and *M. tuberculosis* PPD were unaffected by the addition of peptide, indicating that the peptides were not toxic (data not shown).

Cells from nonimmune donors did not proliferate significantly in response to peptides; the maximum SI recorded was 1.7 at a peptide concentration of 10 µg/ml. Cells from immune donors were tested at two peptide concentrations, 1 and 10 µg/ml, and an SI of >2 was taken as a positive response.

Cells from 16 donors were tested for proliferation in response to nine overlapping 21- to 25-mer peptides representing both dimorphic sequences of MSP-1₁₉ (Table 3). Three of these peptides (peptides 2, 3, and 6) represent conserved sequences, three represent dimorphic sequences of the Wellcome allele (peptides 1, 4, and 5), and three represent dimorphic sequences of the MAD20 allele (peptides 7, 8, and 9).

Cells from 7 of the 16 donors (44%) recognized one or more of the peptides with an SI greater than 2; cells from one donor recognized one peptide with an SI greater than 5. Cells from four donors (donors 1, 7, 14, and 16) proliferated in response to peptides 2 or 6, indicating that they recognized epitopes that are conserved between the two allelic forms of the antigen;

cells from the remaining three donors recognized epitopes that varied between the two alleles and recognized only one of the dimorphic types. The correlation between responses to the synthetic peptides and responses to the recombinant antigens was poor—cells from only two of the seven donors also recognized the recombinant MSP-1₁₉ proteins—suggesting that the epitopes presented by the peptides may differ from those re-

TABLE 3. T-cell proliferative responses to synthetic MSP-1 peptides

Donor no.	SI ^a for T-cell proliferation in response to:								
	WELL/EGF-1 peptide:			WELL/EGF-2 peptide:			MAD20 EGF-1 peptide:	MAD20 EGF-2 peptide:	
	1	2	3	4	5	6	7	8	9
1		2-5			2-5				
2									
3									
4									
5									
6									
7	2-5	2-5	2-5						
8									
9									
10									
11							2-5		2-5
12					2-5				
13					2-5				
14	2-5	2-5			2-5	2-5			
15									
16							>5		

^a The absence of data indicates that no response was observed. SIs were calculated relative to that of the control medium. Counts-per-minute values for control medium alone ranged from 795 to 2,269.

FIG. 2. SDS-PAGE gel of reduced and nonreduced MSP-1₁₉ recombinant proteins. Lanes: 1, molecular mass markers; 2, Well/EGF-1 reduced and alkylated; 3, Well/EGF-1 nonreduced; 4, MAD20 EGF-1 reduced and alkylated; 5, MAD20 EGF-1 nonreduced; 6, MAD20 EGF-2 reduced and alkylated; 7, MAD20 EGF-2 nonreduced; 8, GST reduced and alkylated; 9, GST nonreduced.

sulting from processing of the recombinant antigens. The prevalence of T-cell responses to MSP-1₁₉-derived peptides was almost twice that of responses to MSP-1₁₉ recombinant proteins, suggesting that presentation of the antigen in a denatured (non-disulfide-bonded) form may enhance its recognition.

Effect of secondary structure of MSP-1₁₉ on its recognition by T cells. Since T-cell responses to linear peptides were more prevalent than responses to disulfide-bonded recombinant antigens, we wondered whether the presence of the disulfide bonds hindered antigen processing and presentation. We therefore compared T-cell proliferative responses to reduced and nonreduced recombinant antigens representing both single and double EGF-like domains.

After reduction and alkylation, the dialyzed proteins were run on SDS-PAGE gels; the change in mobility of the reduced proteins (which ran more slowly than the nonreduced proteins) indicated that the disulfide bonds had been disrupted (Fig. 2). We have previously shown that reduction of MSP-1₁₉ by this method destroys the native conformation required for antibody recognition (13). Reduced and nonreduced proteins were tested at a range of concentrations (1 to 10 μ g/ml), and the highest SI for each donor is shown.

Reduction of the GST control protein had no effect on its recognition (gm SI = 5.0 before and 7.12 after reduction, not significantly different). After reduction, proliferative responses to PPD were slightly lower (gm SI = 58.7 before reduction and 32.84 after reduction) but the prevalence of positive responses was not affected. The proliferative response to TT was enhanced in some donors, which is consistent with the known secondary structure of TT, but overall the mean SI was not significantly increased (gm SI = 4.68 before reduction and 4.96 after reduction).

Both the frequency and the intensity of the proliferative response to all the MSP-1₁₉ proteins were higher for reduced proteins than for nonreduced proteins (Table 2). The difference in counts-per-minute values between reduced and nonreduced proteins was highly significant (Wilcoxon signed rank test; $P < 0.05$ in all cases).

The specificity of this effect is demonstrated by the facts that responses to the reduced MAD20 proteins were twice as prevalent as responses to reduced Wellcome proteins (as would be predicted from the known frequencies of the two parasite genotypes in the local population), that the effect was donor specific in that only some donors showed an enhanced response to the antigens after reduction, that those donors who responded most strongly to the reduced protein tended also to show some response to the nonreduced protein, and that cells from European donors did not respond to either reduced or

nonreduced proteins (data not shown). Furthermore, we tested the supernatants of these cultures for the presence of the T-cell-derived cytokine, IFN- γ . IFN- γ responses were enhanced in some donors following protein reduction (Table 4). Cells from 13 of the 19 donors produced IFN- γ in response to nonreduced *M. tuberculosis* PPD; 11 of these also produced IFN- γ in response to reduced *M. tuberculosis* PPD. Actual levels of IFN- γ were similar for reduced and nonreduced *M. tuberculosis* PPD. Cells from only one donor produced IFN- γ in response to nonreduced TT, and cells from one other donor produced it in response to reduced TT. In contrast, in the case of MSP-1-induced IFN- γ , cells from seven donors produced IFN- γ only in response to reduced MSP-1 proteins or the level of IFN- γ production was increased in response to reduced antigen; with cells from four donors the IFN- γ response was abolished by reduction of the antigen. Thus, in the majority of donors, reduction of MSP-1₁₉ antigens appears to enhance the IFN- γ response as well as the T-cell proliferative response.

Antibody responses. (i) Recombinant proteins (Tables 1 and 2). Plasma from 15 of the 35 individuals (43%) possessed antibodies which recognized the Wellcome sequence of MSP-1₄₂. All but one of these individuals also possessed antibodies to at least one of the MSP-1₁₉ proteins, suggesting that much of the antibody reactivity may be to epitopes within the relatively conserved sequence of MSP-1₁₉, with plasma from only one donor (donor 8) appearing to recognize sequences within the MSP-1₃₃ region of the Wellcome MSP-1₄₂ construct. In contrast, 30 of 34 samples tested contained antibodies which bound to the MSP-1₃₃ sequence of the MAD20 allele, confirming that this dimorphic region is naturally immunogenic and that the allele is widely distributed in The Gambia. The discordance between recognition of Wellcome MSP-1₄₂ and MAD20 MSP-1₃₃ suggests that very few individuals have antibodies which recognize conserved epitopes within MSP-1₃₃.

Fourteen of the 35 donors (40%) had antibodies to MSP-1₁₉, and, in most donors, these antibodies seemed to bind to both the Wellcome sequence and the MAD20 sequence. Only nine serum samples recognized the individual EGF-like domains, with eight recognizing the first EGF-like domain and one recognizing the second domain. These data are in close agreement with our previous findings for serorecognition of MSP-1₁₉ in this population (13).

Thus, although antibodies to MSP-1₁₉ tend to be cross-reactive (recognizing both sequences equally), antibodies to MSP-1₃₃ appear to be highly sequence specific.

(ii) Synthetic peptides. ELISAs showed that plasma from only 8 of the 35 (23%) donors had antibodies to any of the synthetic peptides (Table 5). Six of these serum samples recognized peptide 6, which represents a conserved sequence at the very C-terminal end of the second EGF-like domain. None of these six serum samples recognized the recombinant antigen MAD20/EGF-2, which contains exactly the same sequence, suggesting that epitopes within the linear sequence are not presented by the disulfide-bonded antigen. A low level of reactivity of sera with linear peptides was not unexpected because we have previously shown that most MSP-1₁₉-specific antibodies in malaria-immune sera recognize complex, disulfide bond-dependent epitopes (13).

Correlation between T-cell responses and seropositivity. Cells from most individuals appeared to recognize T-cell epitopes in the 33-kDa region and also possessed antibodies to the MAD20 sequence of MSP-1₃₃, indicating that this region of the protein is naturally immunogenic. However, there was poor correlation between the T- and B-cell responses to MSP-1₁₉. Of the nine individuals with a positive T-cell response to MSP-1₁₉ recombinant antigens, only three were seropositive for

TABLE 4. IFN- γ response to reduced and nonreduced recombinant antigens

Antigen ^a	IFN- γ production (U/ml) for donor no.:																			% Positive
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
Control medium																				0
WELL/EGF-1			3	6									4		2					21
WELL/EGF-1 red ^b			3												23					11
MAD20 EGF-1				3									3							16
MAD20 EGF-1 red			3										3		7					16
MAD20 EGF-2																3				0
MAD20 EGF-2 red																				5
WELL 19													2							5
WELL 19 red															8					5
MAD20 19																				0
MAD20 19 red															3	3				11
GST															4			2		11
GST red													6		3					11
PPD ^c	28	9	>40	3		8	16	9	7				10	5		>40	13	31		68
PPD red	10	3	>40				8	17	3				4	11		>40	5	25		58
TT				3																5
TT red															6					5
PHA	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	100

^a Antigen designations are as defined in the footnotes to Table 1.

^b red, reduced.

^c PPD, *M. tuberculosis* PPD.

MSP-1₁₉. Of the seven donors with a positive T-cell response to MSP-1₁₉ peptides, four were seronegative. Considering all T-cell responses to MSP-1₁₉ (to recombinant antigen or synthetic peptide), cells from 15 donors responded but only 5 donors were seropositive.

Interestingly, of the 16 donors tested for T-cell recognition of synthetic peptides (Table 3), the 2 donors with particularly high antibody titers to MSP-1₁₉ (donors 14 and 16) both had cells that appeared to recognize the same T-cell epitope: peptide 6, the conserved sequence from the extreme C terminus of MSP-1₁₉. Whether this observation is a coincidence or a real effect is not clear (the number of individuals involved is too small for any meaningful statistical analysis to be performed), but none of the other donors had cells that recognized this epitope. Cells from only one of the two donors (donor 14) recognized the overlapping peptide (peptide 5), suggesting that the fine specificities of the T-cell epitopes differ between the two donors.

DISCUSSION

We have investigated the immune response to the C terminus of MSP-1 in individuals naturally exposed to *P. falciparum* infection. We have shown that MSP-1₃₃ is naturally immunogenic, with almost all malaria-exposed individuals making antibodies to it. We can also deduce, by a comparison of responses to MSP-1₄₂ and to MSP-1₁₉, that T cells recognize epitopes within MSP-1₃₃ which are conserved between the two allelic sequences, while the B-cell epitopes are dimorphic with little antibody cross-reactivity between the MAD20 and Wellcome sequences. In contrast, MSP-1₁₉ is much less immunogenic; antibody responses are detected in only about 50% of

malaria-immune donors. These antibodies recognize epitopes which are cross-reactive between the two sequences. Very few donors made T-cell proliferative or IFN- γ responses to the recombinant MSP-1₁₉ antigens, and those responses which were seen were of very low magnitude. These data are in broad agreement with our own previous studies (13, 31, 32) and those of Udhayakumar et al. (37), who concluded that immunodominant T-cell epitopes were to be found in conserved sequences within the dimorphic MSP-1₃₃ region. Similar conclusions were drawn from a recent analysis of T-cell responses to PfMSP-1 in *Aotus* monkeys immunized with recombinant MSP-1₄₂ (7). The Wellcome and MAD20 sequences of MSP-1 have approximately 47% identity in the MSP-1₃₃ region (22).

These data provide some support for the hypothesis that the lack of T-cell help may contribute to the low prevalence of anti-MSP-1₁₉ antibodies but suggest that simply incorporating T-cell epitopes from elsewhere in the molecule, as suggested by others (37), may not be sufficient to rectify the situation. All our donors made strong T-cell responses to epitopes within the MSP-1₄₂ fragment, but there was no correlation between these responses and seropositivity to MSP-1₁₉. In addition to measuring T-cell responsiveness by determining proliferation and IFN- γ production, which may not be the best markers for B-cell helper activity, we also attempted to measure secretion of interleukin 4 (IL-4) into cell supernatants by an ELISA. IL-4 levels were below the limit of detection of the assay (37a), possibly suggesting that the cells were of an inappropriate phenotype, but measurements were made only at a single time point (day 6), which may not have been optimal. However, it may be that manipulation of the type of T-cell response is as important as induction of the response. Alternatively, it may be

TABLE 5. Serum antibody responses to synthetic peptides

Donor no.	<i>sA</i> ₄₉₂ ^a for serum antibody response to:								
	WELL/EGF-1 peptide:			WELL/EGF-2 peptide:			MAD20 EGF-1 peptide:	MAD20 EGF-2 peptide:	
	1	2	3	4	5	6	7	8	9
1									
2									
3									
4									
5									
6									
7									
8									
9									
10						0–0.5			
11									
12									
13									
14						0–0.5			
15									
16	0–0.5	0–0.5	0–0.5			0–0.5			
17									
18									
19									
20						0–0.5			
21					0–0.5				
22									
23									
24						0–0.5			0–0.5
25									
26									
27									
28						0–0.5			0–0.5
29									
30									
31		0–0.5							
32									
33									
34									
35									

^a The absence of data indicates that no response was observed.

necessary to maintain a physical link between the T- and B-cell epitopes to ensure that cognate interactions occur; secondary processing of MSP-1₄₂ may destroy some T-cell epitopes or may separate the T-cell epitopes in MSP-1₃₃ from the B-cell epitopes in MSP-1₁₉, thus inhibiting cognate interactions.

T-cell responses to nonreduced MSP-1₁₉ proteins are of low prevalence and intensity; responses to synthetic peptides were approximately twice as prevalent as responses to recombinant proteins. Coincidentally, the MSP-1₁₉ peptides we used were very similar in sequence to those used by Udhayakumar et al. (37) and, interestingly, the patterns of peptide recognition were very similar. Although both the intensity and prevalence of the responses were higher in Kenyan adults than in Gambian adults (which may relate to the higher level of malaria transmission in Kenya), the ranking of the peptides by the frequency of responders was remarkably consistent between the two studies, with peptides 2, 5, and 6 being the most immunodominant and peptides 3 and 8 being the least. This suggests, first, that peptides 2, 5, and 6 may resemble peptides produced by processing of the native protein *in vivo* and, second, that peptides 2, 5, and 6 bind to major histocompatibility complex (MHC) class II molecules that are relatively common in both Kenyan and Gambian populations. Interestingly, in the

present study, cells from the two donors with particularly high titers of antibodies to MSP-1₁₉ both recognized epitopes within overlapping peptides 5 and 6, suggesting that this may be a useful sequence for induction of high-titer responses and long-term memory. Cells from one of these donors also recognized recombinant MSP-1₁₉, further suggesting that this peptide sequence may represent a naturally processed epitope.

In contrast, a recent study looking at T-cell proliferative responses to a panel of synthetic peptides representing various conserved sequences from the N-terminal and central regions of MSP-1 found that only one peptide-specific T-cell clone also responded to native MSP-1 in the form of parasite extracts, suggesting that randomly selected peptides (or even peptides selected on the basis of predicted amphipathicity) may bear little resemblance to naturally processed epitopes (30).

Our data suggest that *in vitro* T-cell responses to recombinant MSP-1₁₉ are enhanced by prior reduction of the antigen, suggesting that the six putative disulfide bonds may form a reduction-resistant unit that withstands lysosomal degradation. Reduction of disulfide bonds has been shown to be an essential step in antigen processing, linearizing the polypeptide and allowing access of proteolytic enzymes which generate the short peptides which bind to MHC molecules for presentation to T cells (9). Reduction and alkylation, by a method essentially the same as that used here, increased MHC class II binding and T-cell recognition of the model antigens hen egg lysozyme and RNase A (9). Disulfide reduction per se, in the absence of proteolysis, may be sufficient to allow proteins to bind directly to MHC class II molecules (21). Further studies are needed to confirm the generality of this finding and to examine the role of disulfide reduction in the processing and presentation of MSP-1.

It is possible that antigen processing *in vivo* is more efficient than it is *in vitro* and that some antigen-presenting cells are more able to cope with proteins with numerous disulfide bonds than are peripheral blood-derived macrophages. If so, T-cell activation by MSP-1₁₉ may be more widespread *in vivo* than is apparent from *in vitro* studies. Further experiments are required to determine if immunization with a mixture of reduced and unreduced MSP-1₁₉ results in more effective priming of the anti-MSP-1₁₉ antibody response than immunization with nonreduced antigen alone.

The near universal recognition of MSP-1₄₂ raises the question of whether such immune responses are in themselves protective. Our initial studies showed that both antibody and T-cell responses to MSP-1₄₂ were significantly associated with resistance to clinical malaria in African children (31). Subsequent dissection of the antibody response to MSP-1₄₂ has indicated that protective epitopes lie within both MSP-1₁₉ (13) and MSP-1₃₃ (12a). In addition, the low prevalence of T-cell responses to MSP-1₁₉ suggests that the protective T-cell responses that we detected in response to MSP-1₄₂ (31) were in fact induced by epitopes within MSP-1₃₃. Taken together, these data suggest that the dimorphic 33-kDa fragment of MSP-1 should be further evaluated as a potential vaccine antigen.

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REFERENCES

- Al-Yaman, F., B. Genton, K. J. Kramer, S. P. Chang, G. S. N. Hui, M. Baisor, and M. P. Alpers. 1996. Assessment of the role of naturally acquired antibody levels to *Plasmodium falciparum* merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity. *Am. J. Trop. Med. Hyg.* **54**:443–448.
- Andersson, G., H. T. Ekre, G. Alm, and P. Perlmann. 1989. Monoclonal antibody two-site ELISA for human IFN γ . *J. Immunol. Methods* **125**:89–96.
- Blackman, M. J., H. 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 cell invasion and is the target of invasion-blocking antibodies. *J. Exp. Med.* **172**:379–382.
- 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 MSP1₃₃ as a non-covalently associated complex with other fragments of the MSP1. *Mol. Biochem. Parasitol.* **50**:307–316.
- 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.
- 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.
- Chang, S. P., S. E. Case, W. L. Gosnell, A. Hashimoto, K. J. Kramer, L. Q. Tam, C. Q. Hashiro, C. M. Nikaido, H. L. Gibson, C. T. Lee-Ng, P. J. Barr, B. T. Yokota, and G. S. N. Hui. 1996. A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects *Aotus* monkeys against malaria. *Infect. Immun.* **64**:253–261.
- 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.
- Collins, D. S., E. R. Unanue, and C. V. Harding. 1991. Reduction of disulfide bonds within lysosomes is a key step in antigen processing. *J. Immunol.* **147**:4054–4059.
- Conway, D. J., B. M. Greenwood, and J. S. McBride. 1992. Longitudinal study of *Plasmodium falciparum* polymorphic antigens in a malaria-endemic population. *Infect. Immun.* **60**:1122–1127.
- Crisanti, A., H. M. Muller, C. Hilbich, F. Sinigaglia, H. Matile, M. MacKay, J. Scaife, K. Beyreuther, and H. Bujard. 1988. Epitopes recognised by human T cells map within the conserved part of gp190 of *Plasmodium falciparum*. *Science* **240**:1324–1326.
- 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.
- Egan, A. 1995. Ph.D. thesis. University of Edinburgh, Edinburgh, United Kingdom.
- Egan, A. F., et al. Submitted for publication.
- Egan, A. F., J. A. Chappel, P. A. Burghaus, J. S. Morris, J. S. McBride, A. A. Holder, D. C. Kaslow, and E. M. Riley. 1995. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1₉, the carboxy-terminal fragment of the major merozoite surface protein of *Plasmodium falciparum*. *Infect. Immun.* **63**:456–466.
- Egan, A. F., J. Morris, G. Barnish, S. Allen, B. M. Greenwood, D. C. Kaslow, A. A. Holder, and E. M. Riley. 1996. Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19kDa C-terminal fragment of the merozoite surface antigen, PfMSP1. *J. Infect. Dis.* **173**:765–769.
- Etlinger, H., P. Caspers, H. Matile, H. Schonfeld, 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.
- 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.* **81**:478–486.
- Guttinger, M., P. Romagnoli, L. Vandel, R. Meloen, B. Takacs, R. J. L. Pink, and F. Sinigaglia. 1991. HLA polymorphism and T cell recognition of a conserved region of p190, a malaria vaccine candidate. *Int. Immunol.* **3**:899–906.
- Hall, R., J. E. Hyde, M. Goman, D. L. Simmons, I. A. Hope, M. MacKay, J. Scaife, B. Merkli, R. Richle, and J. Stocker. 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. *Nature* **311**:379–382.
- Holder, A. A., M. J. Blackman, P. A. Burghaus, J. A. Chappel, I. T. Ling, N. McCallum-Deighton, and S. Shai. 1992. A malaria merozoite surface protein (MSP1)—structure, processing and function. *Mem. Inst. Oswaldo Cruz* **87**(Suppl III):37–42.
- Holder, A. A., R. R. Freeman, and S. C. Nicholls. 1988. Immunisation against *Plasmodium falciparum* with recombinant polypeptides produced in *Escherichia coli*. *Parasite Immunol.* **10**:607–617.
- Jensen, P. E. 1993. Acidification and disulfide reduction can be sufficient to allow intact proteins to bind class II MHC. *J. Immunol.* **150**:3347–3356.
- 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.
- Jongwutiwes, S., K. Tanabe, S. Nakazawa, T. Yanagi, and H. Kanbara. 1992. Sequence variation in the tripeptide repeats and T cell epitopes in P190 (MSA-1) of *Plasmodium falciparum* from field isolates. *Mol. Biochem. Parasitol.* **51**:81–90.
- 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.
- Kumar, S., A. Yadava, D. B. Keister, J. H. Tian, M. Ohl, K. A. Perdue-Greenfield, L. H. Miller, and D. C. Kaslow. 1995. Immunogenicity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. *Mol. Med.* **1**:325–332.
- Ling, I. T., S. A. Ogun, and A. A. Holder. 1994. Immunization against malaria with a recombinant protein. *Parasite Immunol.* **16**:63–67.
- Miller, L. H., T. Toberts, 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.
- Murphy, V. F., W. C. Rowan, M. J. Page, and A. A. Holder. 1990. Expression of hybrid malaria antigens in insect cells and their engineering for correct folding and secretion. *Parasitology* **100**:177–183.
- Peterson, M. G., R. L. Coppel, M. B. Moloney, and D. J. Kemp. 1988. Third form of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. *Mol. Cell. Biol.* **8**:2664–2667.
- Quakyi, I. A., J. Currier, A. Fell, D. W. Taylor, T. Roberts, R. Houghton, R. England, J. A. Berzofsky, L. H. Miller, and M. F. Good. 1994. Analysis of human T cell clones specific for conserved peptide sequences within malaria proteins. *J. Immunol.* **153**:2082–2092.
- Riley, E. M., S. J. Allen, J. Wheeler, M. J. Blackman, S. Bennett, B. Takacs, H. Schönfeld, A. A. Holder, and B. M. Greenwood. 1992. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen of *Plasmodium falciparum* are associated with reduced malaria morbidity. *Parasite Immunol.* **14**:321–338.
- Riley, E. M., S. Morris-Jones, M. J. Blackman, B. M. Greenwood, and A. A. Holder. 1993. A longitudinal study of naturally acquired cellular and humoral immune responses to a merozoite surface protein (MSP1) of *Plasmodium falciparum* in an area of seasonal malaria transmission. *Parasite Immunol.* **15**:513–524.
- 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. Kan. 1987. Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *Proc. Natl. Acad. Sci. USA* **84**:3014–3018.
- Sinigaglia, F., B. Takacs, H. Jacot, H. Matile, J. R. L. Pink, A. Crisanti, and H. Bujard. 1988. Nonpolymorphic regions of p190, a protein of the *Plasmodium falciparum* erythrocytic stage, contain both T and B cell epitopes. *J. Immunol.* **140**:3568–3572.
- Tanabe, K., M. MacKay, M. Goman, and J. Scaife. 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite isolated from *Plasmodium falciparum* merozoites. *J. Mol. Biol.* **195**:273–287.
- Taylor, R. R., A. Egan, D. McGuinness, A. Jepson, R. Adair, C. Drakely, and E. Riley. 1996. Selective recognition of malaria antigens by human serum antibodies is not genetically determined but demonstrates some features of clonal imprinting. *Int. Immunol.* **8**:905–915.
- Udhayakumar, V., D. Anyona, S. Kariuki, Y. P. Shi, P. B. Bloland, O. H. Branch, W. Weiss, B. L. Nahlen, D. C. Kaslow, and A. A. Lal. 1995. Identification of T and B cell epitopes recognized by humans in the C-terminal 42-kDa domain of the *Plasmodium falciparum* merozoite surface protein (MSP)-1. *J. Immunol.* **154**:6022–6030.
- Waterfall, M. Unpublished data.