Dominance of Conserved B-Cell Epitopes of the *Plasmodium falciparum* Merozoite Surface Protein, MSP1, in Blood-Stage Infections of Naive *Aotus* Monkeys

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We have shown that conserved B epitopes were immunodominant in animals hyperimmunized with parasite-purified or recombinant merozoite surface protein MSP1 of *Plasmodium falciparum*. Cross-priming studies also suggested that a conserved T-helper epitope(s) is efficient in inducing the anti-MSP1 antibody response. In this study, we determined whether a similar profile of immune responses was induced during live *P. falciparum* infections. Naive *Aotus* monkeys were infected by blood-stage challenge with either one of the two dimorphic MSP1 alleles represented by the FUP and FVO parasites. Sera collected after parasite clearance were analyzed by enzyme-linked immunosorbent assays (ELISAs). Monkeys infected with parasites carrying one allelic form of MSP1 had antibodies that were equally reactive with homologous or heterologous MSP1s. This preferential recognition of conserved epitopes of MSP1 was confirmed by competitive binding ELISAs. Studies with *Plasmodium yoelii* and *P. falciparum* show that the C-terminal 19-kDa fragment of MSP1, MSP1₁₉, is the target of protective immunity. Thus, monkey sera were assayed for recognition with recombinant MSP1₁₉s expressing variant and conserved B epitopes. Results of direct and competitive binding ELISAs showed that the anti-MSP1₁₉ antibodies were also directed primarily against conserved determinants. The similarities between vaccine- or infection-induced antibody responses suggest a possible reciprocal enhancement of the two populations of anti-MSP1 antibodies when a subunit MSP1 vaccine is introduced into populations living in areas where malaria is endemic. This together with previous observations that conserved determinants are important in MSP1-mediated immunity provides an optimistic outlook that a subunit MSP1 vaccine may be effective and practical for field applications in malaria-exposed populations.

The C-terminal region of the merozoite surface protein, MSP1, of *Plasmodium falciparum* is a proteolytically processed 42-kDa polypeptide, MSP1₄₂, which is further cleaved to form a 19-kDa protein, MSP1₁₉, on the invading merozoites (1, 13). Immunization with MSP1₄₂ or MSP1₁₉ can elicit a protective immune response against *P. falciparum* as well as the rodent malaria parasite, *Plasmodium yoelii* (4, 9, 22, 23). Results from in vitro and passive transfer studies show that humoral antibodies play, at least in part, a crucial role in MSP1-mediated protection (1–3, 5, 10, 28). The target(s) of the protective antibodies is the C-terminal 19-kDa epidermal growth factor-like domains of MSP1, MSP1₁₉ (2, 10, 23). The C-terminal region of *P. falciparum* MSP1 consists of conserved and non-conserved epitopes (26, 27, 29, 30). Nonconserved regions possess either one of two allelic sequences of the dimorphic MSP1 alleles (29) or variant epitopes (8, 25). Previous studies have shown that hyperimmunization of animals with intact MSP1 or the C-terminal 42-kDa fragments primarily induced antibodies

to conserved B epitopes at the C-terminal 19-kDa region, MSP1₁₉ (14–17). While these studies suggest that conserved determinants are immunodominant, the interpretation must be taken in the context of the experimental conditions, i.e., vaccine-induced immunity under hyperimmunization with a strong adjuvant, namely, complete Freund's adjuvant (14–17). Whether a similar dominance of conserved B epitopes occurs following live malarial infection remains to be determined. This is of importance in terms of the strategies for vaccine design, particularly if the vaccine's efficacy relies on natural exposure for optimal boosting and if the targeted populations are individuals living in malarious areas.

Malarial infections in naturally exposed humans are often composed of mixed parasite clones (7, 21). These observations pose difficulties in determining the dominance of immune responses to conserved epitopes relative to those to nonconserved epitopes, since antibodies against multiple variants and allelic B epitopes in a mixed infection are difficult to distinguish from antibodies specific for conserved determinants. This may be further complicated if the past histories of infection (symptomatic or asymptomatic) are unclear. We took an alternate approach by analyzing the antibody responses in naive *Aotus* monkeys receiving a blood-induced infection of *P. falciparum* parasites carrying either one of the two allelic forms

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TABLE 1. Inhibition of binding of MSP1₁₉ variant-specific MAbs to MSP1₁₉ in the presence of different MSP1₁₉ variants

Variant-specific anti-MSP1 ₁₉ MAb	% Inhibition		
	E-KNG ^a	Q-KNG	E-TSR
111.4 ^b	7	84	9
4H4/34 ^c	5	0	77

^a MSP1₁₉ variant used as inhibitor (5 µg/ml).

^b Specific for the Q variant and assayed for binding to Q-KNG. Maximum OD without inhibitors was 0.755; antibody concentration was 0.5 µg/ml.

^c Specific for the TSR variant and assayed for binding to E-TSR. Maximum OD without inhibitors was 0.391; antibody concentration was at a 1:50 dilution of ascitic fluids.

of MSP1. Postchallenge sera from these monkeys were used to study the antibody binding to native MSP1 of the two dimorphic alleles (15) as well as three recombinant polypeptides corresponding to the C-terminal 19-kDa epidermal growth factor-like domains of MSP1, MSP1₁₉. These recombinant polypeptides possess different variant B-cell epitopes known within the 19-kDa region (20). Results showed that antibody responses to the C-terminal region of MSP1 during live *P. falciparum* infections are directed primarily against conserved B epitopes. The implications of these results in terms of vaccine design are discussed.

MATERIALS AND METHODS

***Aotus* monkeys and *P. falciparum* infections.** Naive *Aotus nancymai* (karyotype 1) monkeys were infected by blood-stage challenge with FUP (Hawaii) or FVO (DNAX) isolate parasites (6). The challenge inocula were 10⁶ and 10⁵ infected erythrocytes, respectively. For the FUP-infected monkeys, peak parasitemias ranged from 29,000 to 1,184,000/µl; the duration of patency ranged from 9 to 21 days. For the FVO-infected monkeys, peak parasitemias ranged from 68,000 to 580,000/µl; the duration of patency ranged from 8 to 20 days. Monkeys either cured the infections spontaneously or were drug treated to terminate the infections. Sera were obtained just prior to challenge and approximately 6 weeks postchallenge for antibody assays. Seven monkeys from each group (i.e., FUP infected or FVO infected) were used for antibody analyses. The genotypes of the MSP1 proteins of the FUP and FVO parasites with respect to clonality and C-terminal allelic and variant types were confirmed by PCR and variant-specific monoclonal antibodies (MAbs) as described previously (15, 17, 24).

Antibody assays. Serum antibody specific for MSP1 antigens was assayed by direct binding and competitive binding enzyme-linked immunosorbent assays (ELISAs), as described previously (15). To standardize ELISAs with MSP1 polypeptides, different antigens were plated at concentrations which gave equivalent optical densities (ODs) by use of MAb 5.2 directed against a conserved epitope at the C-terminal 19-kDa region of MSP1. *Aotus* sera were assayed at a 1:100 dilution, and antibody responses were compared by use of OD readings (at 410 nm) at this dilution. End point titers were not used for analyses of responses because preliminary ELISA screening showed that the anti-MSP1 titers in these monkeys were low ($\leq 1:1,000$). Background binding levels (measured as ELISA ODs) of the prechallenge sera to purified MSP1s and recombinant MSP1₁₉s were 0.022 ± 0.016 and 0.029 ± 0.016 , respectively. For inhibition ELISAs, the final dilution of monkey sera was 1:200. Murine MAbs 111.4 and 4H4/34, specific for the Q and TSR variant epitopes of MSP1₁₉, respectively (1, 8, 20), were used as controls to illustrate the specificities of inhibition ELISAs for the MSP1₁₉ variants (Table 1).

MSP1 antigens for ELISAs. Parasite MSP1s were isolated from FUP and FVO in vitro cultures by MAb affinity chromatography (with MAb 5.2) as described previously (15). The C-terminal 19-kDa epidermal growth factor-like domains of MSP1 were expressed as three different polypeptides, each possessing the combination of variant-specific B-cell epitopes (1, 8, 20) identified to date (23). Variant epitopes on the 19-kDa fragments of FUP and FVO parasites are represented in these recombinant polypeptides (20). The yeast-expressed polypeptides were purified on Ni-nitrilotriacetic acid agarose as described previously (20).

RESULTS

Antibody responses to allelic forms of MSP1. Sera from *Aotus* monkeys infected with FUP parasites were assayed for binding to FUP and FVO parasite MSP1s (Fig. 1A). The two MSP1s have been shown previously to differ in most of their allelic sequences (15). As shown, sera reacted equally well with either the FUP MSP1 or the FVO MSP1. Similar findings were obtained with sera from monkeys infected with FVO parasites (Fig. 1B). In the FVO-infected and FUP-infected *Aotus* monkeys, the proportion of anti-MSP1 antibodies specific for conserved versus that specific for nonconserved epitopes was studied by use of competitive binding ELISAs. Figure 2 shows that in FUP-infected monkeys, the binding of antibodies to FUP MSP1 was inhibited equally by FVO MSP1 and FUP

FIG. 1. Binding of serum samples from seven *Aotus* monkeys infected with FUP parasites (A) and seven *Aotus* monkeys infected with FVO parasites (B) to purified FUP MSP1 and FVO MSP1 as measured by ELISAs. *Aotus* serum samples were assayed at a 1:100 dilution.

FIG. 2. Inhibition ELISAs. Inhibition of binding of serum samples (at a 1:200 final dilution) from three representative FUP-infected *Aotus* monkeys (A to C) to FUP MSP1-coated plates by use of soluble FUP MSP1 and FVO MSP1 as inhibitors. Maximal ELISA ODs in the absence of inhibitors were 0.444 (A), 0.504 (B), and 0.387 (C).

FIG. 3. Inhibition ELISAs. Inhibition of binding of serum samples (at a 1:200 final dilution) from three representative FVO-infected *Aotus* monkeys (A to C) to FVO MSP1-coated plates by use of soluble FUP MSP1 and FVO MSP1 as inhibitors. Maximal ELISA ODs in the absence of inhibitors were 0.460 (A), 0.525 (B), and 0.409 (C).

FIG. 4. Binding of serum samples from seven *Aotus* monkeys infected with FUP parasites (A) and seven *Aotus* monkeys infected with FVO parasites (B) to the recombinant MSP1₁₉ variants E-KNG, Q-KNG, and E-TSR. *Aotus* serum samples were assayed at a 1:100 dilution.

MSP1. Both the maximum percentages of inhibition and the 50% inhibition concentrations were similar for the two MSP1s. In the reciprocal assays, the binding of antibodies from FVO-infected monkeys to FVO MSP1 was inhibited equally by FUP-MSP1 and the homologous inhibitor FVO-MSP1 (Fig. 3). These data suggest that allelic B-cell determinants of MSP1 are not immunodominant in live, blood-stage infections in *Aotus* monkeys. In addition, since the variable or strain-specific tripeptide repeat regions (block 2) of the two MSP1 proteins have been shown to be nonhomologous (15), our data also suggest that the variable repeat regions (epitopes) of MSP1s are not immunodominant in live malarial infections.

Antibody responses to different recombinant MSP1₁₉ variants. A recent vaccination study of *Aotus* monkeys shows that the C-terminal 19-kDa fragment of *P. falciparum* MSP1,

MSP1₁₉, confers protection against blood-stage challenge (22). Previous studies using MAbs have defined two variant B-cell epitopes within the epidermal growth factor-like domains of MSP1₁₉ (1, 8). These are the KNG versus TSR and the Q versus E variants. Sera from FUP-infected and FVO-infected monkeys were assayed for binding to three recombinant MSP1₁₉s bearing these variants, namely, E-KNG (FUP isolate), Q-KNG (FVO), and E-TSR (3D7). As shown in Fig. 4, the binding levels of antibodies from FVO-infected monkeys (or FUP-infected monkeys) to all of the MSP1₁₉ variants were similar. This is further confirmed by competitive binding ELISAs as shown in Fig. 5 and 6. The binding levels of antibodies from FUP-infected monkeys to the MSP1₁₉ variant (E-KNG) specific for the FUP MSP1 were equally as inhibited by heterologous MSP1₁₉ variants (Q-KNG and E-TSR) from the FVO and 3D7 parasites, respectively, as they were by the homologous MSP1₁₉ inhibitor E-KNG. Similar results were obtained with sera from FVO-infected monkeys assayed for binding to Q-KNG, in the presence of Q-KNG, E-KNG, or E-TSR as an inhibitor (data not shown). These data suggest that in malaria infections of naive *Aotus* monkeys, the antibodies that are specific for the MSP1₁₉ fragment are directed primarily against conserved determinants on this molecule.

The 70 to 80% maximal inhibition observed in the assays may not be due to background ODs since prechallenge sera showed minimal binding. Aggregation of inhibitors may occur at high inhibitor concentrations, thereby lowering the inhibition efficiency. Analyses of the levels of anti-MSP1 antibodies in monkeys and the courses of infection showed that there was no correlation between antibody levels and peak parasitemia or the duration of patent infections (data not shown).

DISCUSSION

Immune responses to a particular antigen during live infections with the pathogen may be different from those induced by artificial or adjuvant-assisted immunizations. Murine antibody responses to the NANP repeat epitope of *Plasmodium berghei* circumsporozoite protein (CSP) are restricted to a few *H*-2 haplotypes; however, this genetic nonresponsiveness was not observed during infections of mice with live sporozoites (12). In contrast, mice experiencing live infections with the nematode parasite *Ascaris suum* show an *H*-2-restricted response to the 14-kDa excretory-secretory antigen, while immunization of mice with the purified protein in complete Freund's adjuvant can overcome this genetic restriction (31). In herpes simplex virus type 1, immunization of mice with the protective glycoprotein D induced T cells that preferentially recognize a single dominant T-cell epitope on glycoprotein D (34), while mice infected with the live virion have T cells specific for an additional 21 epitopes spanning the entire glycoprotein D molecule (34). The T-cell epitope specificity to *P. berghei* CSP is also different between mice immunized with recombinant CSP and mice that received live infections of irradiated sporozoites (33). The potentially disparate responses between live infections and artificial immunizations are of particular concern for vaccine development such as that with the *P. falciparum* MSP1 antigen. One concern is that immunization of the hosts that have preexisting, natural immune responses may fail to induce the desired protective specificities observed in naive hosts. This may be a result of epitope suppression induced under concurrent live infections. A second concern is that the requirement of postimmunization exposure to live infections for optimal boosting of a protective immune response may render a subunit vaccine much less effective as a

FIG. 5. Inhibition ELISAs. Inhibition of binding of serum samples (at a 1:200 final dilution) from four representative FUP-infected *Aotus* monkeys (A to D) to plates coated with the MSP1₁₉ variant E-KNG by use of soluble MSP1₁₉ variants E-KNG and Q-KNG as inhibitors. Maximal ELISA ODs in the absence of inhibitors were 0.794 (A), 0.560 (B), 0.667 (C), and 0.749 (D).

result of the induction of non-cross-reactive T-helper determinants.

Studies have indicated that the recognition of conserved determinants are of key importance in MSP1-mediated immunity. Inhibition of *P. falciparum* parasite growth by polyclonal antibodies and MAbs specific for MSP1 was directed against a conserved epitope(s) (15, 17, 28). Monkeys hyperimmunized with MSP1 were protected from heterologous parasite challenge (11). We have shown that conserved regions of MSP1 were dominant in antibody responses induced via hyperimmunizations (15, 17). More recently, cross-priming studies with heterologous MSP1s suggested that conserved T-helper determinants are efficient in inducing antibody responses to MSP1s (16). The results of the present study show that conserved B epitopes were also dominant in *Aotus* monkeys experiencing live malaria infections. Since all animals were challenged for the first time with *P. falciparum* malaria, the antibody profile resembled more closely a primary immune response. Thus, the dominance of conserved B epitopes observed in the present study reflects the intrinsic immunogenicity of these determinants. This should be distinguished from the possibility that conserved epitopes may be subdominant;

but, as a result of repeated infections with or exposures to non-cross-reactive parasite variants, antibodies to these epitopes may be selectively boosted to constitute the majority of the anti-MSP1 antibodies.

A recent seroepidemiological study using sera from adults living in areas of Kenya where malaria is hyperendemic shows that among the 64 human serum samples tested for binding to the MSP1₁₉s, 24 of these samples (approximately 38%) failed to react with one or more of the three MSP1₁₉ variants, i.e., E-KNG, E-TSR, and Q-KNG (32). In contrast, similar surveys of antibody responses in both children and adults from areas of the Philippines where malaria is hyperendemic (Palawan District) as well as hypoendemic (Morong District) showed that the overwhelming majority (95 to 97%) of the serum samples recognized all of the MSP1₁₉ variants tested (13a). Differences in the antibody profiles between these two studies may be due to dissimilarities in transmission characteristics or simply to differences in assay methods or conditions. Nevertheless, there is evidence that human antibody responses may approximate the immunological profiles observed in *Aotus* monkeys.

The lack of correlation between antibody titers to MSP1 and

FIG. 6. Inhibition ELISAs. Inhibition of binding of serum samples (at a 1:200 final dilution) from four representative FUP-infected *Aotus* monkeys (A to D) to plates coated with the MSP1₁₉ variant E-KNG by use of the soluble MSP1₁₉ variants E-KNG and E-TSR as inhibitors. Maximal ELISA ODs in the absence of inhibitors were 0.989 (A), 0.559 (B), 0.475 (C), and 0.833 (D).

peak parasitemias in monkeys observed in this study is not surprising since it has been demonstrated that high titers of MSP1-specific antibodies are prerequisites for biological activities (18, 19). It is unlikely that the very low titers of antibodies observed in these monkeys during primary infections will have protective value.

The protective epitope(s) on *P. falciparum* MSP1 has yet to be precisely identified. However, a small fragment, MSP1₁₉, at the C-terminal region has been shown recently to elicit protection in *Aotus* monkeys (22). It is noteworthy that *Aotus* monkeys protected by hyperimmunization with MSP1 produced anti-MSP1₁₉ antibodies that primarily recognized a conserved region(s) on MSP1₁₉ (20). Such dominance of anti-MSP1₁₉ response to conserved epitopes on MSP1₁₉ was also a characteristic feature in naive *Aotus* monkeys experiencing live malaria infections. The similarities between vaccine- and infection-induced antibody responses observed in this study provide evidence for a possible reciprocal enhancement of the two populations of anti-MSP1 antibodies when a subunit MSP1 vaccine is introduced into populations living in areas where malaria is endemic. Along this line, epitope mapping of natural immune responses to *P. falciparum* MSP1, particularly to the

C-terminal protective fragment, should have a more direct relevance to the design of MSP1 vaccines. The next step is to identify relevant conserved epitopes that are immunogenic in both natural exposure and vaccine-induced immune responses and to demonstrate that immunization with recombinant MSP1s containing such epitopes will lend itself to natural boosting.

Our present findings together with previous observations that conserved determinants on MSP1 are important in MSP1-mediated immunity (11, 15, 17) provide an optimistic outlook that a subunit MSP1 vaccine based on conserved epitopes may be effective and also practical for field applications in malaria-exposed populations.

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