MAJOR ARTICLE

Identification of a Conserved Region of *Plasmodium falciparum* MSP3 Targeted by Biologically Active Antibodies to Improve Vaccine Design

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Merozoite surface protein 3 (MSP3) is a target of antibody-dependent cellular inhibition (ADCI), a protective mechanism against *Plasmodium falciparum* malaria. From the C-terminal half of the molecule, 6 overlapping peptides were chosen to characterize human immune responses. Each peptide defined at least 1 non–cross-reactive B cell epitope. Distinct patterns of antibody responses, by level and IgG subclass distribution, were observed in inhabitants of a malaria-endemic area. Antibodies affinity purified toward each peptide differed in their functional capacity to mediate parasite killing in ADCI assays: 3 of 6 overlapping peptides had a major inhibitory effect on parasite growth. This result was confirmed by the passive transfer of anti–MSP3 antibodies in vivo in a *P. falciparum* mouse model. T helper cell epitopes were identified in each peptide. Antigenicity and functional assays identified a 70–amino acid conserved domain of MSP3 as a target of biologically active antibodies to be included in future vaccine constructs based on MSP3.

The asexual blood-stage multiplication of the malarial parasite is responsible for the acute symptoms of malaria in humans. Epidemiological observations have shown that adults living in endemic areas, although they are constantly reinfected and frequently carry parasites, control their levels of parasitemia and show substantial clinical resistance, compared with children [1]. Repeated infections and continued exposure to the parasite are required to reach this level of immunity against disease [2]. This state of naturally acquired immunity against disease, a phenomenon that is called premunition [3], is not a sterile immunity and is marked by chronic low-grade parasitemia without clinical symptoms.

The passive transfer of serum IgG from clinically immune individuals has been shown to be able to control disease and the level of parasitemia in nonprotected individuals who are exposed to geographically diverse parasite strains [4–6]. We have earlier shown that the protection afforded by IgG has no major direct effect on parasite invasion and growth in red blood cells (RBCs)—rather, it acts in association with blood monocytes through an antibody-dependent cellular inhibition (ADCI) mechanism that inhibits parasite development [7]. The cytophilic nature of protective IgG has been established [8, 9], and the importance of these antibodies in protection against malaria has also been demonstrated in other independent studies [10, 11].

Our search for the targets of the protective antibodies, using ADCI as a functional assay, led us to identify merozoite surface protein 3 (MSP3) as one such target [12]. MSP3 is associated with merozoite surface molecules, possibly through the coiled-coil structures that have been predicted to be formed by the heptad repeats and the C-terminal leucine zipper domain [13]. The N-terminal part of the molecule consists of regions that are polymorphic among different strains. In contrast, the C-terminal part of the molecule is highly conserved among the various isolates of the parasite [14, 15], and it is this region that was earlier identified by screening of a *Plasmodium falciparum* expression library by use

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of functional ADCI assays [12]. Previous studies of MSP3 have focused only on a 27-aa region (aa 184–210, corresponding to the 3D7 strain, MSP3b) of the C-terminal part, which was earlier identified as a target of protective antibody response in hyperimmune serum samples [12].

We decided to further characterize the antigenicity of other regions in the C-terminal part of the molecule. Six overlapping peptides were designed (MSP3a, MSP3b, MSP3c, MSP3d, MSP3e, and MSP3f), each of which represented a different region of the conserved C-terminal part of the molecule. They were used to analyze the naturally occurring immune responses in individuals from the malaria-endemic village of Dielmo, Senegal, and their potential relationship to protection from malaria disease. The functional role of human antibodies specific to each region was assessed under in vitro conditions in the ADCI assay and was further confirmed by passive transfer in vivo in an immunodeficient mouse model grafted with *P. falciparum*–infected human RBCs [16, 17].

This process led us to identify a 70-aa region of MSP3 as the target for naturally occurring protective antibody responses. This region thus defines the minimal domain essential for the design of any vaccine construct based on MSP3.

MATERIALS AND METHODS

Antigens. MSP3 recombinant protein constructs and peptides were designed on the basis of the *P. falciparum* 3D7 strain sequence (NCBI protein_id, NP_700818.1). Two recombinant hexahistidine-tagged proteins, MSP3-NTHis$_{21–184}$ and MSP3-CTHis$_{191–354}$, were purified as described elsewhere [18]. The 6 peptides (MSP3a$_{167–191}$, MSP3b$_{184–210}$, MSP3c$_{203–230}$, MSP3d$_{211–252}$, MSP3e$_{275–307}$, and MSP3f$_{302–354}$) correspond to the conserved region of MSP3 C-terminal region. A small region (aa 253–274; 72% glutamic acid) was excluded from this analysis because glutamate-rich antigenic determinants exhibit cross-reactivity among several different *P. falciparum* antigens [19]. The peptides were synthesized according to standard peptide synthesis procedures [20].

Human serum and lymphocyte samples. For the affinity purification of antibodies specific to each MSP3 region, we used serum samples from 30 hyperimmune individuals from Ivory Coast that had been previously used for passive-transfer experiments in Thai patients with malaria and were found to be effective in controlling disease and parasitemia [6].

For immunoepidemiological studies, we used plasma samples from 48 permanent residents of the village of Dielmo, Senegal, who had various degrees of exposure to malaria (age, 3.5–53.4 years; mean age, 13.1 ± 1.8 years; mean stay in the village, 707/730 days of follow-up). In this region, malaria transmission is intense and perennial (~200 infected mosquito bites/person/year); over the course of 2-year period, the mean number of malaria attacks was 2.4 ± 5.4 episodes/person. Nineteen individuals had no malaria attack (mean age, 15.7 ± 3.1 years), whereas 29 individuals had at least 1 malaria attack (mean age, 11.4 ± 2.2 years) during the next 2 years. All inhabitants of Dielmo were actively monitored by medical doctors on a daily basis for febrile episodes, and those due to malaria were accurately diagnosed as described elsewhere [21]. This allowed us to examine the pattern of the IgG isotype response toward different regions of MSP3 in individuals who were clearly distinguishable as "protected" (no malaria attack) or "nonprotected" (⩾1 malaria attack) during the 2-year follow-up period of the study. This group was representative of the whole village in terms of age distribution, with respect to occurrence or absence of malaria attack.

Mononuclear cells obtained from inhabitants of Dielmo were transported within 4 h to laboratories in Dakar and used for T cell proliferation and the determination of interferon (IFN)–γ against MSP3a, MSP3b, and MSP3c peptides, according to methods described elsewhere [22, 23]. In brief, the proliferative responses of the cells were assessed in quadruplicate in 96-well round-bottomed plates (Nunclon; Nunc) by incubation for 6 days at 37°C in 5% CO_2 in the presence of each peptide used at 10 μg/mL, followed by the addition of 1 μCi of [^{3}H]thymidine overnight and counting of the incorporated radioactivity in a liquid scintillation counter. Unstimulated cultures served as negative controls, and purified protein derivative and phytohemagglutinin were used as positive controls. The IFN-γ concentration in pooled supernatants from quadruplicate wells was assessed by a capture ELISA performed in duplicate, by use of the anti–human IFN-γ monoclonal antibody (MAb) 350B10G6 and biotin-labeled MAb 67F12A8 (Biosource) for coating and revealing, respectively, according to the manufacturer's instructions. The reaction was revealed by use of streptavidin–horseradish peroxidase and tetra-methyl benzidine chromogen, and the optical density was measured at 450 nm. For practical reasons, mainly the number of cells available per donor, the other 3 peptides used for antibody assays could not be included in T cell assays. Lymphoproliferation studies were performed with samples from 61 inhabitants (29 female and 32 male; mean age, 27.31 years), and IFN-γ secretion was studied in 31 inhabitants (19 female and 12 male; mean age, 33.94 years). The 3 peptides proved to induce no significant response in peripheral blood mononuclear cells from 16 control, non–malaria-exposed donors (data not shown), which indicated that they had no mitogenic or superantigenic effect.

ELISA. The ELISA was performed for the detection of total IgG and subclasses, as described elsewhere [8, 9]. Monoclonal mouse anti–human subclasses IgG1–IgG4 (clones NL16 [Boehringer], HP6002 [Sigma], Zg4 [Immunotech], and RJ4 [Immunotech]) were selected for their affinity and reactivity for African allotypes and were used as secondary antibodies at dilutions of 1:2000, 1:5000, 1:5000, and 1:1000, respectively.

The specific reactivity of each serum sample was obtained by subtracting the optical density value of a control protein (0.25 μg of bovine serum albumin/well) from that of the test antigens. For calculating the threshold of significance of antibody responses, a set of 8 randomly selected serum samples from individuals never exposed to malaria was tested against each antigen, as controls. Results were expressed as the ratio of the mean optical density from test serum samples to the mean optical density of control subset + 3× the SD of the control serum samples. Serum samples were considered to be positive for ratios $\geqslant$1.

Affinity purification of antibodies. Because the ADCI assay requires the cooperation of antibodies with the Fc-γ RII receptor [7], a group of 30 hyperimmune serum samples from individuals from Ivory Coast were first screened for IgG subclass distribution against different MSP3 peptides and recombinants. Serum samples were selected for the affinity purification of antibodies against any given MSP3 construct on the basis of their high reactivity against that region, with minimal reactivity toward the adjacent peptides and a high content of cytophilic IgG antibodies (IgG1 and IgG3). Independent serum pools (each of which was made up of 5–7 individual serum samples) were used to affinity purify antibodies to different regions of MSP3. The ratios of cytophilic to noncytophilic IgG subclasses (IgG1 + IgG3:IgG2 + IgG4) of the serum pools used were estimated to be 9.56 for MSP3NT, 4.25 for MSP3CT, 1.29 for MSP3a, 3.86 for MSP3b, 1.29 for MSP3c, 4.58 for MSP3d, 1.59 for MSP3e, and 3.68 for MSP3f. Previous studies have shown that the profile of cytophilic antibodies observed in affinity-purified antibodies was similar to that of the serum sample pool used for affinity purification (S. Singh, S. Soe, and P.D., unpublished data).

Affinity purification was done as described elsewhere [24], by use of a 2.5% aqueous suspension of polystyrene beads (mean diameter, 10 μm; Polysciences) to coat the peptides or recombinant proteins. Specific antibodies were eluted by use of 0.2 mol glycine/L (pH 2.5) and were immediately neutralized to pH 7.0 by use of a 2 mol/L aqueous Tris solution. Affinity-purified antibodies were dialyzed extensively against PBS followed by RPMI and were concentrated by use of Centricon concentrators (Millipore), filter sterilized, and, after the addition of 1% albumax (Gibco BRL), stored at 4°C. Affinity-purified antibodies were used at a concentration of 10 μg/mL in ELISA to ascertain their specificity.

Immunofluorescence assay (IFA). Because the ability of the antibodies to recognize the native parasite protein is the critical factor in biological assays, IFA was used to adjust the concentration of affinity-purified antibodies. IFA was performed on air-dried, acetone-fixed, thin smears of *P. falciparum* mature schizonts, as described elsewhere [25], to assess the binding activity of affinity-purified antibodies to the parasite protein. The effective concentration of each antibody was adjusted to a 1:200 IFA end-point titer for use in functional assays.

Functional in vitro antibody assays. The antibody-dependent, monocyte-mediated ADCI assays were performed in duplicate by use of laboratory-maintained strains 3D7 and Uganda Palo-Alto, as described elsewhere [7]. Monocytes from healthy, non–malaria-exposed donors were prepared as described elsewhere [7]. The affinity-purified antibodies, adjusted to a concentration yielding a 1/200 IFA end-point titer, were added at a rate of 10 μL in 90 μL of complete culture medium, which yielded a final titer of 1/20 in the ADCI assay. After cultivation for 96 h, the level of parasitemia was determined on Giemsa-stained thin smears from each well by the microscopic examination of $\geqslant$50,000 erythrocytes. Monocyte-dependent parasite inhibition is expressed as the specific growth inhibition index (SGI): SGI $= 1 -$ ([percentage of parasitemia with monocytes and test IgG/percentage of parasitemia with test IgG)/(percentage of parasitemia with monocytes and normal IgG/percentage of parasitemia with normal IgG]) $\times$ 100. Although the SGI calculation takes into account a possible direct antiparasite effect of monocytes, because this is observed with 10%–15% of monocyte preparations, we excluded them as an additional precaution.

Passive immunization of* P. falciparum*–infected immunocompromised mice. The use of the *P. falciparum*–human RBC (HuRBC)–Beige-Xid-Nude (BXN) mouse model for assessing the effect of antibodies on different blood-stage antigens of *P. falciparum* has been detailed elsewhere [16]. In brief, 6–8-week-old male BXN mice (Charles River Laboratories), manipulated under pathogen-free conditions, were treated with liposomes that contained dichloromethylenediphosphonate (Roche Diagnostics) and antipolymorphonuclear neutrophil MAb NIMP-R14 (NIMR), to reduce their innate immune response. *P. falciparum*–infected human RBCs were injected intraperitoneally (ip) on day 0, and uninfected RBCs were injected at 4-day intervals. The level of blood parasitemia was examined microscopically. Mice with stable parasitemia (0.1%–1%) were injected ip with 3×10^6 human peripheral blood monocytes positively selected by $CD14^+$ magnetic beads (MACS; Miltenyi Biotech), followed 24 h later by the injection of 3×10^6 monocytes together with 200 μL of affinity-purified antibodies to MSP3 at a 1:200 IFA end-point titer, as described above. Nonspecific esterase staining [7] showed that >98% of $CD14^+$ cells were monocytes.

Statistical analysis. Univariate analysis was performed by use of the Mann-Whitney *U* test. Fisher's exact test was used for the contingency analysis. The association between the risk of malaria attack and the level of antibodies was tested with JMP software (SAS Institute), by use of a stepwise regression model in which we controlled for the confounding effect of

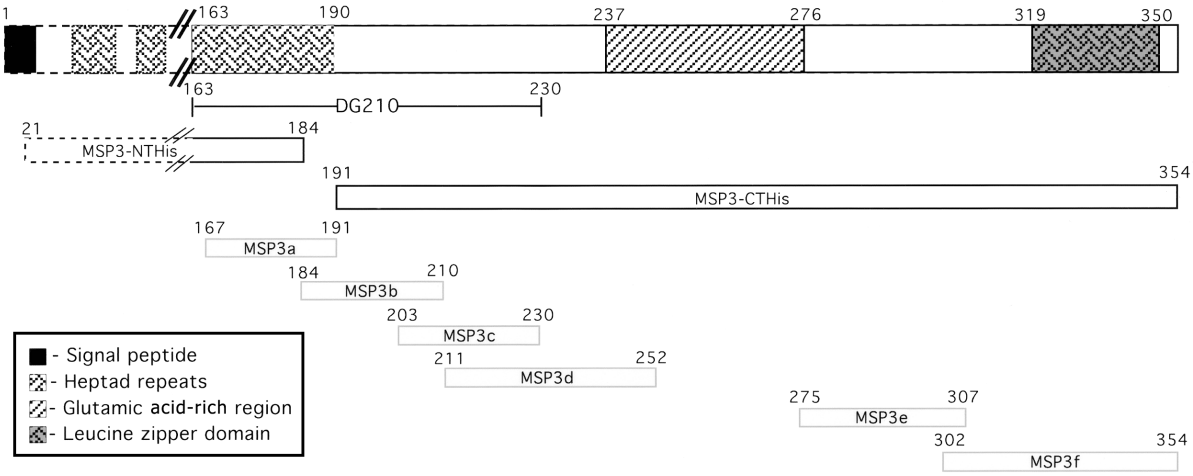


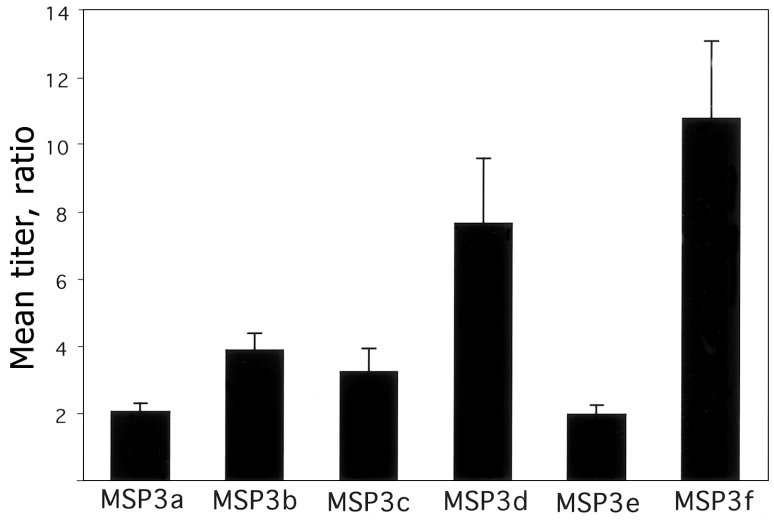
Figure 1. Schematic presentation of *Plasmodium falciparum* merozoite surface protein 3 (MSP3) and the design of MSP3 recombinant proteins (MSP3-NTHis and MSP3-CTHis) and peptides (MSP3a, MSP3b, MSP3c, MSP3d, MSP3e, and MSP3f). The representation of the N-terminal part of MSP3 is compressed here *(dotted line).* DG210 represents the λgt11 expression clone originally identified as the target of protective antibodies [8]. The nos. show amino-acid positions for each region on the basis of the sequence derived from 3D7 strain.

age. The analysis of variance was applied to the regression model. The test of the null hypothesis was based on the variance ratio denoted by *F*, and departures from the null hypothesis tended to give values of *F* that were greater than unity.

RESULTS

Non–cross-reactive B cell epitopes defined by each of the 6 MSP3 C-terminal peptides. IgG responses were measured against different regions of the MSP3 C terminal (figure 1) in a group of 30 hyperimmune serum samples from individuals from Ivory Coast. As shown in figure 2, there were differences in the levels and prevalence of IgG toward each region, but antibody responses were detected against each of the C-terminal peptides.

Antibodies were then affinity purified from selected hyperimmune serum samples specific to each peptide and examined for their reactivity against the other peptides. In this way, it



	MSP3a	MSP3b	MSP3c	MSP3d	MSP3e	MSP3f
% prevalence	30	53.33	50	63.33	33.33	76.66

Figure 2. Total IgG response against different regions of merozoite surface protein 3 (MSP3) in hyperimmune serum samples ($n = 30$) from individuals from Ivory Coast, used to prepare protective IgG for passive-transfer experiments in humans [6]. Antibody reactivity was considered to be positive if the ratio of the mean optical density of the test serum samples to the mean optical density of control serum samples + 3 × the SD of the control serum sample was ⩾1. The figure represents the mean antibody titer (expressed as ratio) of positive serum samples against each region. The table shows the percentage prevalence of positive serum samples reactive to different regions of MSP3, in terms of total IgG.

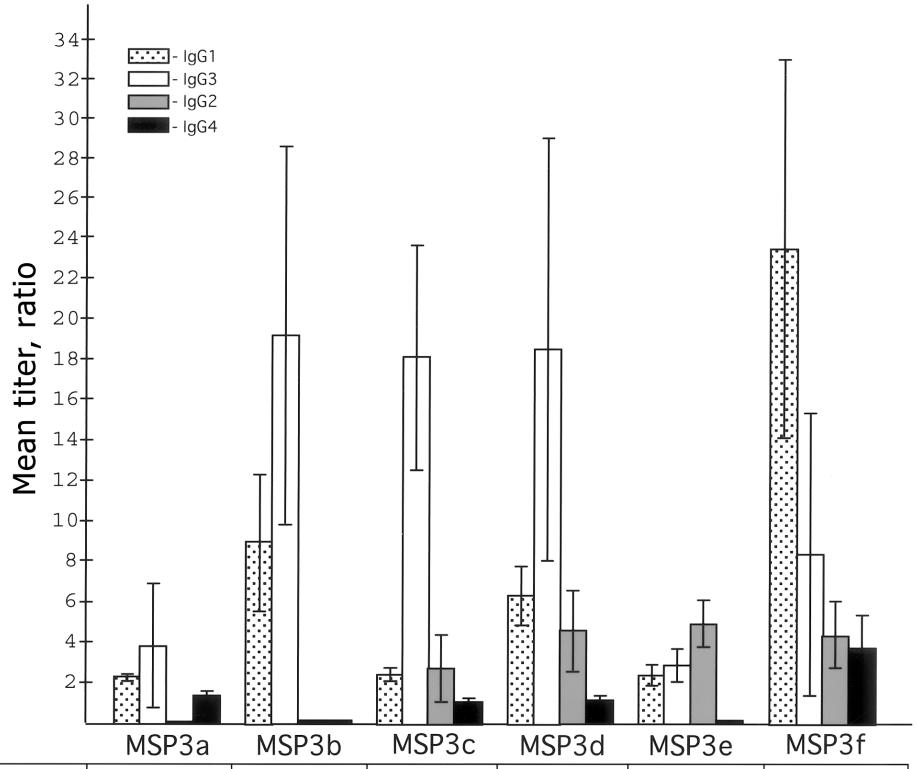
Table 1. Specificity of affinity-purified human anti–merozoite surface protein 3 (MSP3) antibodies, as determined by ELISA.

Antibody	MSP3a	MSP3b	MSP3c	MSP3d	MSP3e	MSP3f
Anti-MSP3a	**0.78**	0.09	0.08	0.08	0.09	0.08
Anti-MSP3b	0.05	**1.11**	0.09	0.08	0.09	0.07
Anti-MSP3c	0.07	0.10	**1.04**	0.09	0.09	0.08
Anti-MSP3d	0.10	0.08	0.16	**1.01**	0.09	0.09
Anti-MSP3e	0.08	0.08	0.08	0.08	**0.95**	0.10
Anti-MSP3f	0.07	0.07	0.08	0.08	0.10	**0.92**

NOTE. Mean optical density values at 450 nm from duplicate wells are shown. All the peptides were used under identical coating conditions. Bold type represents positive reactivity.

was possible to affinity purify antibodies that were specific to each peptide but did not show cross-reactivity with other regions (table 1). These observations indicated that each of the peptides covering the MSP3 C terminal defines at least 1 B cell epitope that does not share antigenic determinants with other regions. Each of the affinity-purified antibodies was also found to be positive in IFAs of *P. falciparum* asexual blood stages, which indicates that antipeptide antibodies were reactive with the native parasite protein (data not shown).

Distinct isotype patterns of the IgG response toward different MSP3 peptides. We analyzed plasma from 48 individuals, 3–53 years old, from the endemic village of Dielmo, Senegal, to study the distribution and pattern of IgG isotype response against the different regions of the C-terminal part of MSP3 defined by the peptides. As shown in figure 3, both the level of antibody response and the pattern of IgG isotype were distinct against each region. The prevalence of responders varied for each region of MSP3 (6.25%–60.41% for IgG1, 4.16%–47.91% for IgG3, 0%–10.41% for IgG2, and 0%–12.5% for IgG4). We found that antibodies to MSP3a and MSP3e were less prevalent, and, when they were present, they were detected only at low levels. Antibodies to MSP3b, MSP3c, MSP3d, and



% prevalence	MSP3a	MSP3b	MSP3c	MSP3d	MSP3e	MSP3f
IgG1	6.25	14.58	22.91	29.16	8.33	60.41
IgG3	4.16	29.16	47.91	35.41	8.33	6.25
IgG2	0	0	8.33	10.41	4.16	2.08
IgG4	6.25	0	10.41	12.5	0	8.33

Figure 3. Prevalence and mean titer of antibodies against different regions of merozoite surface protein 3 (MSP3) in serum samples ($n = 48$) from Dielmo, Senegal. Antibody reactivity was considered to be positive if the ratio of the mean optical density of test serum samples to the mean optical density of control serum samples + 3 × the SD of the control serum samples was ⩾1. The figure represents antibody titers (expressed as a ratio) of the positive serum samples against each region. The table shows the percentage prevalence of positive serum samples reactive to different regions of MSP3, in terms of IgG isotype.

MSP3f were the most prevalent and were predominantly of cytophilic subclasses. Among the cytophilic isotypes, IgG3 reactivity was found to be predominant against MSP3b, MSP3c, and MSP3d. By contrast, IgG1 reactivity against MSP3f was stronger and more prevalent than that against IgG3. This suggests that the antibody response elicited to any region of MSP3 was not dependent on a response to other regions.

It had been observed earlier that the cytophilic IgG response plays an important role in protection against malaria [8–11]. We further addressed the relationship between clinical protection that had been monitored on a daily basis and the pattern of isotype responses observed against each peptide. In the present study, protection was defined as the absence of any clinical malaria attack during the 2 years after plasma samples were obtained. Higher IgG3 titers against MSP3b, MSP3c, and MSP3d were observed among protected, compared with nonprotected, subjects. An association between the levels of IgG3 antibodies directed to MSP3b and MSP3d and protection from occurrence of malaria attack ($P = .037$ and .057, respectively) was observed. In the case of MSP3c, this association did not reach statistical significance; however, levels of anti–MSP3c IgG3 antibodies were twice as high in individuals who did not develop malaria, compared with those who did. The association between levels of IgG1 and protection against malaria attack was observed to be significant for MSP3d ($P = .025$), and a similar trend was observed for MSP3b ($P = .328$), but not for MSP3c. Neither IgG1 nor IgG3 responses to MSP3f were found to be associated with protection. IgG2 and IgG4 antibody responses against different regions of MSP3 were detected only at low levels and were not found to be associated with protection.

In a further step, a multivariate stepwise regression analysis was performed to control for age, by use of dichotomous variables of both antibody response (responders or nonresponders) and occurrence of malaria attack (protected or nonprotected). A significant association of protection with IgG3 antipeptide responses was observed against 3 of 6 peptides—MSP3b ($F = 4.98$, $P = .025$), MSP3c ($F = 3.02$, $P = .082$), and MSP3d ($F = 6.57$, $P = .01$)—but not against the other 3.

Inhibition of parasite growth by naturally occurring antibodies against MSP3b, MSP3c, and MSP3d in functional in vitro ADCI assays. To assess the function of naturally occurring human antibodies to different regions of MSP3 in ADCI assays, each affinity-purified antibody was adjusted to a concentration that yielded the same reactivity to the native parasite protein. Results (figure 4) showed that the level of parasite inhibition elicited by antibodies against the recombinant proteins MSP3NT and MSP3CT were comparable to that observed for the pool of African IgG (PIAG), which was used elsewhere for a passive-transfer experiment in humans [6].

Anti–MSP3b, –MSP3c, and –MSP3d affinity-purified antibodies were found to exert a strong monocytemediated antiparasitic activity in ADCI that was comparable to that of antibodies against MSP3CT and PIAG, whereas anti–MSP3a and –MSP3f antibodies were not found to have parasite inhibitory activity (figure 4). Anti-MSP3e antibodies showed only marginal antiparasite activity that was slightly higher than the threshold level of significance. Results were reproducible among 4 independent ADCI assays. At the concentrations used, none of the abovementioned antibodies showed the direct inhibition of parasite growth.

Strong reduction of* P. falciparum *parasitemia by anti–MSP3b and anti–MSP3d antibodies in a humanized mouse model. The observation from the in vitro ADCI assays that anti–MSP3b, –MSP3c, and –MSP3d antibodies were strongly effective at inhibiting parasite growth was further assessed in vivo by use of the *P. falciparum*–HuRBC-BXN mouse model. The value of this new mouse model for studying the in vivo effect of human antibodies and antimalarial drugs on the blood-stage growth of *P. falciparum* has been recently documented [16, 17]. We chose to study antibodies to MSP3d and MSP3f, which were positive and negative in ADCI, respectively, compared with anti–MSP3b antibodies, which we used as positive controls, whose antiparasitic effect has already been demonstrated [16].

As seen in figure 5, the level of parasitemia increased and reached a plateau over the next 3 weeks. The injection of anti–MSP3f antibodies with human monocytes did not affect parasite growth, in agreement with the results of the in vitro ADCI assays. In the other 2 mice, the injection of human monocytes alone on day 22 did not affect parasite growth, in keeping with earlier observations [16]. The injection of affinity-purified anti–MSP3b or –MSP3d human antibodies on day 23 resulted in a sharp decrease in parasitemia. The passive transfer of anti–MSP3b antibodies resulted in the clearance of parasites. The passive transfer of anti–MSP3d antibodies resulted in a decrease of parasitemia >95% (figure 5). Thus, results from the in vivo passive transfer in this mouse model confirmed in vitro results and further validated the functional antiparasite activity of naturally occurring antibodies against the 70-aa region covered by peptides MSP3b and MSP3d.

T cell responses against MSP3 peptides in malaria-exposed individuals. T lymphocyte responses could be studied against only 3 (MSP3a, MSP3b, and MSP3c) of 6 C-terminal peptides in inhabitants from Dielmo, Senegal, because of practical limitations in field. The proliferative response, which was determined by use of peripheral blood lymphocytes from 61 individuals (age range, 1–84 years; mean age, 27.34 years) showed that the prevalence of T helper cell responders was 16.4% against MSP3a, 28% against MSP3b, and 21.3% against MSP3c, respectively. IFN-γ secretion, which was monitored in 31 of these individuals, showed that the prevalence of IFN-γ responders was 42% against MSP3a, 55% against MSP3b, and 61.3% against MSP3c. These results indicate that each of the

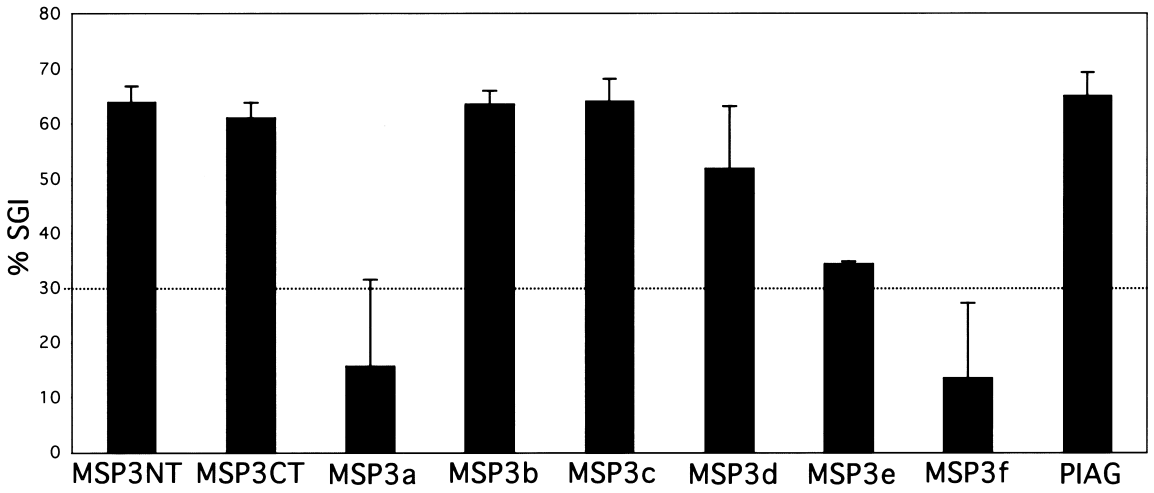


Figure 4. Effect of affinity-purified human antibodies on parasite growth in antibody-dependent cellular inhibition assay. The histograms represent mean values of the percentage of the specific growth inhibition index (SGI; as explained in the text) from 2 independent experiments ± SE; values >30% are significant. PIAG, positive control IgG from the pool of serum samples from adults from Ivory Coast used for passive-transfer experiments in humans [6].

3 MSP3 peptides tested defines at least 1 T cell epitope. In addition, IFN-γ secretion results suggested that at least some of the responding cells belonged to the Th1-like type.

DISCUSSION

In the search for candidates for a malaria vaccine, we focused our studies on antigens targeted by the most potent immunity—that acquired over the years by individuals living in hyperendemic areas. We have described that this premunition is mediated by IgG that is active through an indirect mechanism, which implicates monocytes. We then used ADCI to identify MSP3 as a target of protective IgG [12]. The present study was aimed at characterizing antigens within the conserved C terminus of MSP3 and evaluating the function and biological effects of the corresponding antibodies.

Indeed, the C-terminal half of the molecule, starting from the third heptad repeat, is highly conserved in the different isolates tested so far [14, 15], whereas the N-terminal half of MSP3 shows an overall dimorphism (3D7-like and K1-like) [14, 15]. Therefore, we decided to focus on the C-terminal region, because part of it (DG210; figure 1) was identified to be a target of protective human antibodies in our initial study

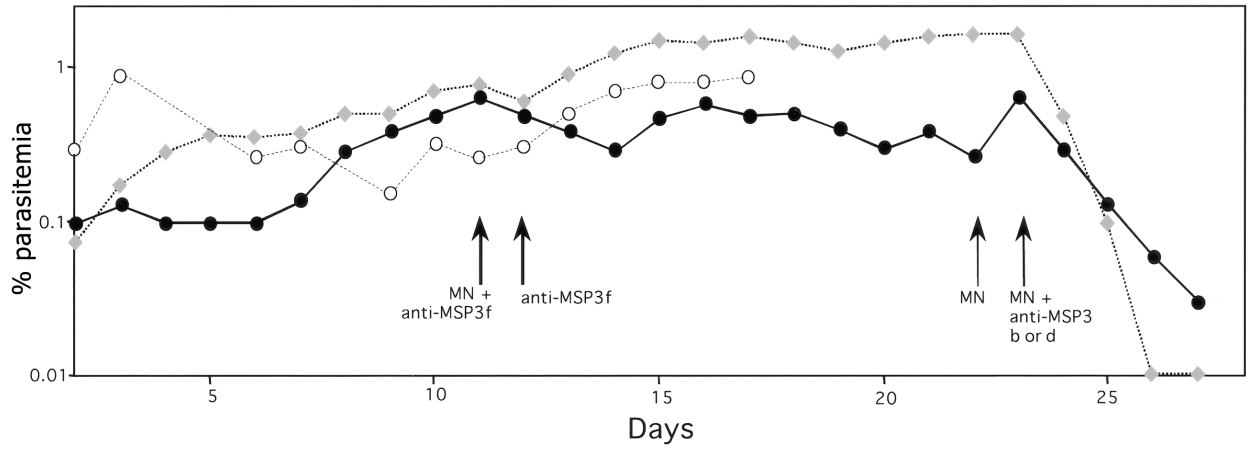


Figure 5. In vivo transfer of affinity-purified human anti–merozoite surface protein 3 (MSP3) antibodies, together with human peripheral blood monocytes in *P. falciparum*–human red blood cell–Beige-Xid-Nude mice. The curves show the course of parasitemia as determined by microscopic examination of thin blood smears from mice injected with anti–MSP3b antibodies *(gray diamonds),* anti–MSP3d antibodies *(black circles),* or control anti–MSP3f antibodies that were ineffective in the antibody-dependent cellular inhibition assay *(white circles). Arrows,* days at which injections were made, first of human monocytes (MNs) and then followed by monocytes together with anti–MSP3 antibodies (200 μL; immunofluorescence assay titer, 1:200).

[12] and because antigen conservation is a critical criterion for the successful development of a malaria vaccine.

Using 6 overlapping synthetic peptides covering the conserved C-terminal half of MSP3, we have shown that antibody patterns to each region differ markedly in terms of prevalence, titer, isotype distribution, association with clinical protection, and antiparasitic activity in vitro and in vivo. Antibody titers against MSP3a and MSP3e were lower than those of the remaining 4 peptides. Responses to MSP3b, MSP3c, MSP3d, and MSP3f were mostly of cytophilic IgG subclasses—predominantly of IgG1 isotype against MSP3f and IgG3 isotype against the others. A similar difference in subclass response to distinct regions of a single protein has been reported for MSP1 [26]. These observations suggest that IgG class switching involved during the maturation of the antibody response toward different regions of the MSP3 C terminal is regulated independently. The factors that regulate the maturation of antibodies are not well understood but would be influenced by the nature of the antigen in conjunction with contact-dependent signals from T cells, particularly the cytokines they secrete [27]. Recent observations, however, have suggested that the nature of the malaria antigen might be the major factor that determines antibody subclass [28], which seems to be the case in our study.

The availability of very detailed clinical information, which is a major characteristic of the setup in the village of Dielmo, Senegal, led us to address subclass patterns in relation to protection against the occurrence of malaria. Taking into account the confounding effect of age, we observed that IgG3 responses to MSP3b, MSP3c, and MSP3d were significantly associated with protection. These results are in agreement with those of independent studies that involved larger sample sizes [29] (C. Oeuray, C.R., J.L. Pérignon C. Muller-Graf, A. Tall, C. Rogier, J.F. Trape, and P.D., unpublished data), which have shown an association between the IgG3 response against MSP3b and protection from malaria. For other merozoite surface vaccine candidates, a skewing toward the IgG3 antibody response has been reported for MSP2 in various ethnic groups and under different conditions of malaria transmission [30, 31]; this could be correlated with clinical immunity to malaria [32]. Similarly, the antibody response to the polymorphic block 2 region of MSP1, which has been identified as a target of immunity to clinical malaria, is also skewed toward the IgG3 subclass [33]. However, at least in the latter case, the mechanism of action of these antibodies remains elusive, because it is generally assumed that biologically active anti–MSP1 antibodies are directed to the C-terminal part of the antigen [34].

In contrast, in the present study, the use of functional in vitro ADCI assays provided information about the antiparasitic, biological activity of antibodies toward various regions. Because they were performed under conditions that allowed comparisons, they demonstrated critical differences in antibodies that target different regions of MSP3. It is of interest that very different approaches led to similar conclusions—that is, the in vitro ADCI assays pointed to the importance of exactly the same peptides (MSP3b, MSP3c, and MSP3d) as those indicated by the immunoepidemiological studies. The reasons for this lack of effect of antibodies to MSP3a and MSP3f remains to be investigated. In the case of MSP3f, it is possible that antibodies might not access this epitope on the merozoite surface, because the leucine-zipper domain forms coiled-coil interactions with other molecules [13, 14].

The reliability of in vitro findings could also be confirmed under in vivo conditions [16]. On passive transfer in *P. falciparum*-infected mice grafted with human monocytes and with long-lasting stable parasitemia, anti–MSP3b and –MSP3d antibodies were found to be effective in reducing the *P. falciparum* parasite load.

The vaccine potential of MSP3 was recently confirmed by the protection elicited against *P. falciparum* challenge in *Aotus nancymai* monkeys immunized with full-length MSP3 in Freund's adjuvant [35]. This observation is in agreement with our epidemiological and biological findings. However, the present study has provided additional information derived from the analysis of human immune responses for the design of future vaccine constructs. Indeed, the N terminal of MSP3, although able to induce antibody with functional activity in ADCI, is of debatable value, because of its polymorphism. Furthermore, its inclusion could divert the immune response away from the important conserved region. Within the C-terminal part, the region MSP3e–f was also found to be less valuable, because of the low prevalence and low levels of antibody response to MSP3e and anti–MSP3f antibodies devoid of biological effect. Each of the 3 peptides (MSP3a, MSP3b, and MSP3c) investigated proved to define a non–cross-reactive T cell epitope for populations in endemic areas. Recent vaccine trials performed using the construct defined in the present study confirmed this finding and designated the peptide MSP3d as an additional T cell–epitopic region (R. Audran, M. Cachat, F. Lurati, S. Soe, O. Leroy, G.C., P.D., and F. Spertini, unpublished data).

In summary, the results of immunoepidemiological studies and functional assays led us to define a 70-aa region of the MSP3 molecule. We found that antibodies with antiparasitic effect develop against this region, which covers MSP3b–MSP3d, in humans who have been naturally exposed to malaria. This information is of practical value for future clinical trials for the rational design of subunit vaccine constructs derived from MSP3.

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