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Natural Immune Response to the C-Terminal 19-Kilodalton Domain of *Plasmodium falciparum* Merozoite Surface Protein 1

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We have characterized the natural immune responses to the 19-kDa domain of merozoite surface protein 1 in individuals from an area of western Kenya in which malaria is holoendemic. We used the three known natural variant forms of the yeast-expressed recombinant 19-kDa fragment that are referred to as the E-KNG, Q-KNG, and E-TSR antigens. T-cell proliferative responses in individuals older than 15 years and the profile of immunoglobulin G (IgG) antibody isotypes in individuals from 2 to 74 years old were determined. Positive proliferative responses to the Q-KNG antigen were observed for 54% of the individuals, and 37 and 35% of the individuals responded to the E-KNG and E-TSR constructs, respectively. Considerable heterogeneity in the T-cell proliferative responses to these three variant antigens was observed in different individuals, suggesting that the 19-kDa antigen may contain variant-specific T epitopes. Among responses of the different isotypes of the IgG antibody, IgG1 and IgG3 isotype responses were predominant, and the prevalence and levels of the responses increased with age. We also found that a higher level of IgG1 antibody response correlated with lower parasite density among young age groups, suggesting that IgG1 antibody response may play a role in protection against malaria. However, there was no correlation between the IgG3 antibody level and protection. Furthermore, we observed that although the natural antibodies cross-reacted with all three variant 19-kDa antigens, IgG3 antibodies in 12 plasma samples recognized only the E-KNG and Q-KNG constructs and not the E-TSR antigen. This result suggests that the fine specificity of IgG3 antibodies differentiates among variant-specific natural B-cell determinants in the second epidermal growth factor domain (KNG and TSR) of the antigen.

Malaria remains one of the major public health problems in tropical countries, affecting 300 million to 500 million people and causing 2 million to 3 million deaths per year (10). Vaccination has been considered as an approach that will complement other strategies for the prevention and control of this disease. So far, several stage-specific vaccine candidate antigens of *Plasmodium falciparum* have been sequenced and immunologically characterized. Among these antigens, merozoite surface protein 1 (MSP-1) of the asexual blood stage has emerged as a promising candidate antigen (20). This protein is synthesized as an approximately 200-kDa precursor protein at the schizont stage and is further proteolytically cleaved into a number of discrete products residing on the surface of the merozoite, most of which disappear at the time the merozoite invades the erythrocyte (12). Processing of MSP-1 at the C-terminal region produces a 42-kDa fragment, which is further cleaved to form 33-kDa and 19-kDa fragments. Only the C-terminal 19-kDa fragment of MSP-1 remains on the merozoite surface during erythrocyte invasion and therefore is an ideal target for blocking parasite invasion into the erythrocyte (2).

Gene sequencing of MSP-1 from laboratory and field isolates of *P. falciparum* has revealed that this protein is dimorphic and can be divided into 17 blocks consisting of five conserved, five semiconserved, and seven variable regions (24). It has been predicted that at least six disulfide bonds are formed in the 19-kDa fragment, which creates a folded three-dimensional structure containing two epidermal growth factor (EGF)-like domains (3). Although the C-terminal 19-kDa fragment is highly conserved compared with other regions of the MSP-1 antigen, major variation in four amino acid residues has been found to exist in laboratory and field isolates (15, 16, 19). One of these residues is located in the first EGF-like domain at position 1644, where it has been found to be either Q or E. In the second EGF-like domain, amino acid residues at positions 1691, 1700, and 1701 have been found to be either TSR or KNG. One previous study has revealed that a single amino acid difference (E or Q) in the first EGF-like domain of the 19-kDa region influences the binding of monoclonal antibodies to this domain (6).

There is considerable evidence that the MSP-1 19-kDa antigen of *P. falciparum* is the target of protective immune response. Several in vitro studies have shown that polyclonal and monoclonal antibodies recognizing 19-kDa fragments of *P. falciparum* MSP-1 inhibit parasite invasion and development (2, 5–7). An in vivo study has also demonstrated that immunization with the recombinant 19-kDa homologous structure of MSP-1 (containing both EGF-like domains) from the rodent malaria parasite *Plasmodium yoelii yoelii* protects mice against lethal challenge (8). More recently, the yeast-expressed recombinant 19-kDa fragment of *P. falciparum* MSP-1 ($YMSP1_{19}$ fragment) containing four major amino acid substitutions was produced in a yeast (*Saccharomyces cerevisiae*) expression system and has been shown to retain native conformation (17). It was demonstrated that this $YMSP1_{19}$ antigen possesses a majority of the protective B epitopes of the C-terminal 42-kDa

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region (14). A recent vaccine study using aotus monkeys (*Aotus nancymai*) and $YMSP1_{19}$ fragments was shown to induce protection against parasite challenge (18).

The protective efficacy of a malaria vaccine antigen will ultimately be tested in human vaccine trials. In preparation for evaluating the efficacy of the vaccine in field trials, it is essential to investigate the natural immune response to the candidate vaccine antigen and to determine the association between the specific immune response and protection against malaria. One recent study conducted in Gambia showed that there was an apparent increase in total immunoglobulin G (IgG) levels to the 19-kDa antigen of *P. falciparum* MSP-1 with age. However, the predominant IgG1 isotype response to this antigen did not vary among persons of different ages (9). Our previous study in Kenya revealed that although T-cell proliferative responses to the peptides representing the MSP-1 19-kDa antigen were low, a high proportion of Kenyan adults had antibodies that recognize variants of the MSP-1 19-kDa recombinant protein (25). The present study was designed to (i) characterize the profile of T-cell proliferative responses and IgG isotype responses to the MSP-1 19-kDa recombinant antigen, (ii) examine the relationship between natural antibody isotype responses to the MSP-1 19-kDa antigen and age-dependent protection against malaria, and (iii) evaluate the cross-reactivities of the natural immune response and the three known variant forms of the MSP-1 19-kDa antigen.

MATERIALS AND METHODS

Study site and subjects. The residents of 15 villages located near Lake Victoria (Asembo Bay) in a holoendemic area of western Kenya were randomly chosen for this study. Informed consent was obtained from all the study participants. Transmission is highest during and following the long rains (March to May), with a second peak of transmission occurring after the short rains in October to December. Entomologic inoculation rates as high as 300 infected bites per year have been measured in this area (1). Plasma samples were obtained from 117 individuals aged 2 to 74 years during September 1994 and May 1995. These samples were stored at −20°C until they were used for IgG isotype analysis. Of the 117 individuals, 57 individuals aged >15 years were tested for T-cell proliferative responses to the antigens. Ten to fifteen milliliters of venous blood from each individual was collected aseptically in heparinized tubes and transported to the laboratory in cold conditions within 2 h for a T-cell proliferation assay. Thick blood films were made for all subjects at the time of sample collection and stained with Giemsa stain for examination of malaria parasites. None of the 117 individuals had axillary temperatures of >37.5°C. Persons with parasitemia (≥2,500 parasites per μl) were treated with pyrimethamine-sulfadoxine (Fansidar).

Recombinant protein. So far, the *P. falciparum* parasites bearing the MSP-1 19-kDa variant sequences E-TSR, E-KNG, Q-KNG, and Q-TSR have been identified in laboratory strains and field isolates (15, 16, 19). In our molecular epidemiological study, we have found that the E-TSR, E-KNG, and Q-KNG variants are present in this study area of western Kenya (23a). $YMSP1_{19}$ fragments representing the three known natural variant forms in Kenya were used to measure lymphocyte proliferative responses and IgG antibody isotype responses in this study. The purification and characteristics of the $YMSP1_{19}$ fragments have been described elsewhere (17).

Lymphocyte proliferation assay. RPMI 1640 medium (GIBCO-BRL, Grand Island, N.Y.) supplemented with 5% fetal bovine serum, 5% normal AB^+ human serum (donors not previously exposed to malaria), 2 mM glutamine, and 100 U of penicillin and streptomycin (each) per ml was used. Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) by centrifugation. Briefly, a 50-ml tube containing 10 ml of Ficoll-Hypaque was loaded with 10 to 15 ml of donor blood and centrifuged at 1,500 rpm for 30 min at room temperature. The clear plasma remaining on top of the cells was collected and stored at −70°C for IgG isotype studies. The PBMCs at the interphase were collected, washed three times in 0.85% normal saline, and resuspended in culture medium. Viable PMBC counts were made with a phase-contrast microscope by the trypan blue dye exclusion test. A total of 200,000 PBMCs in 100 μl of culture medium was added to each well of a flat-bottom, 96-well plate (Costar, Cambridge, Mass.). To this, two different concentrations of $YMSP1_{19}$ antigen (0.1 μg/ml and 1 μg/ml) were added in 100 μl of culture medium. Purified protein derivative of tuberculin (Statens Seruminstitut, Copenhagen, Denmark) and phytohemagglutinin (Sigma Chemical Co., St. Louis, Mo.) were used as positive controls in all of the experiments. Each stimulus was tested in quadruplicate. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 5 days and labeled overnight with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci [74.0 GBq]/mmol; DuPont NEN Research Products, Boston, Mass.). Cultures were harvested with a Skatron Harvester, and the radioactivity was determined in a liquid scintillation counter. The geometric counts per minute for each set of quadruplicate wells were calculated, and the stimulation index (SI) was determined as the geometric mean count per minute for $YMSP1_{19}$ antigen-stimulated culture divided by the geometric mean count per minute for the unstimulated medium culture (background). The background count per minute varied between 39 and 1,735 cpm for 55 subjects. The geometric mean of the background count was 285 cpm. Two individuals had background counts of >5,000 cpm. Fifteen blood donors to the Centers for Disease Control blood bank who had no history of travel to malarious regions were used as the normal control group.

IgG isotype titration by enzyme-linked immunosorbent assay (ELISA). To measure IgG isotype antibody levels against $YMSP1_{19}$ fragments, 250 ng of the antigens per ml in borate-buffered saline (167 mM borate–134 mM NaCl [pH 8.0]) was used to coat Dynatech Immunolon plates. After overnight coating at 4°C, the plates were washed, blocked with 5% nonfat lyophilized milk and 0.05% bovine serum albumin suspended in HSPBS-T (0.15 M phosphate buffer [pH 7.4] with 500 mM NaCl and 0.05% Tween 20), and washed with HSPBS-T to remove unbound proteins. Plasma samples were diluted by twofold increments, starting with a 1:100 dilution in HSPBS-T containing 5% milk and 0.1% Nonidet P-40, and allowed to react with $YMSP1_{19}$-coated plates for 1 h at room temperature. The unbound antibodies were washed off with HSPBS-T, and then the plates were incubated with 100 μl of peroxidase-conjugated mouse anti-human IgG isotypes (ICN Biomedical, Inc., Costa Mesa, Calif.) at a 1:500 dilution in PBS-T-M (0.01 M phosphate-buffered saline [pH 7.4] containing 0.05% Tween and 5% milk) for 1 h. After four washes with PBS-T, the plates were incubated with 150 μl of 3,3′,5,5′-tetramethylbenzidine (microwell peroxidase substrate system; KPL Laboratories, Gaithersburg, Md.) for 15 min for color development; the reaction was stopped with 50 μl of 1 M H_3PO_4. The color was read at 450 nm with a Titertek Multiskanread (Flow Laboratories Inc., McLean, Va.). Standard curves with positive controls were made for each set of experiments to calibrate the system. Sixty serum samples from donors with no previous exposure to malaria were obtained from the Centers for Disease Control blood bank. The normal serum samples were assayed at a 1:100 dilution. The mean optical density (OD) plus three standard deviations of the normal group serum samples was used as the cutoff to determine the endpoint titer of antibody response.

Competitive inhibition assay. Competitive inhibition experiments were conducted to determine antibody cross-reactivity among the three variant $YMSP1_{19}$ antigens. A plasma sample from an immune individual was diluted to the 50% antibody binding point and incubated with different concentrations of the three $YMSP1_{19}$ antigens for 1 h at 37°C. Then, antibody reactivity to the E-KNG antigen in the antigen-plasma mixture was determined by ELISA as described above. In all experiments, a 5× NANP (asparagine-alanine-asparagine-proline) repeat of the circumsporozoite protein of *P. falciparum* was used as a negative control inhibitor. The results are presented in terms of percent inhibition (I), which was calculated with the formula $I = \{[OD(0) - OD(X)]/OD(0)\} \times 100$, in which OD(0) is the OD of the tested sample without variant antigen incubation and OD(X) is the OD of the sample incubated with the variant antigen.

Statistical analysis. Statistical analysis was performed with SAS and Epi Info software packages. The chi-square test for trend and Spearman's rank correlation coefficient were used for analysis of the increase of antibody isotype response with age. Multiple regression was used to examine the relevance of IgG isotype response to age-dependent protection against malaria. The association between antibody titers of individual plasma samples and antibody recognition of the three $YMSP1_{19}$ variants was assessed with the Pearson correlation coefficient. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Lymphocyte proliferative response to $YMSP1_{19}$ antigens. (i) Profile of lymphocyte proliferative response. PBMCs from 57 individuals from western Kenya were tested in a lymphoproliferation assay to determine the ability of $YMSP1_{19}$ antigens to induce a proliferative response. There was variation in the pattern of response to different concentrations (0.1 μg/ml and 1 μg/ml) of the $YMSP1_{19}$ antigen in different individuals. While a lower concentration of antigen (0.1 μg/ml) was sufficient to induce a positive response in some individuals, a higher concentration of antigen (1 μg/ml) was needed to elicit a response in other individuals. Therefore, individuals who showed an SI of >2.5 for at least one of the two concentrations of antigen were scored as positive responders. Fifty-four percent of the individuals had a positive proliferative response to the Q-KNG $YMSP1_{19}$ antigen, and 37 and 35% of the individuals responded to the E-KNG and E-TSR $YMSP1_{19}$ constructs, respectively. The SI values for positive responders (Fig. 1) were predominantly in the range of 2.5 to 10; however, the

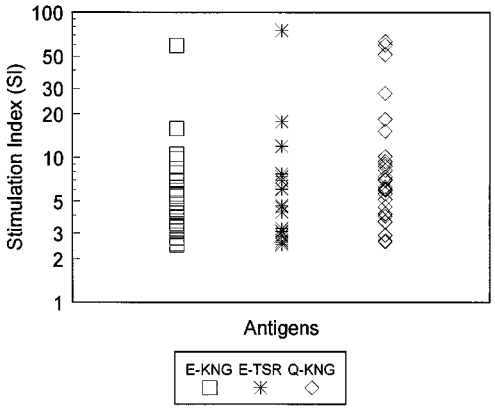

FIG. 1. Magnitude of positive proliferative responses to three variant $YMSP1_{19}$ antigens.

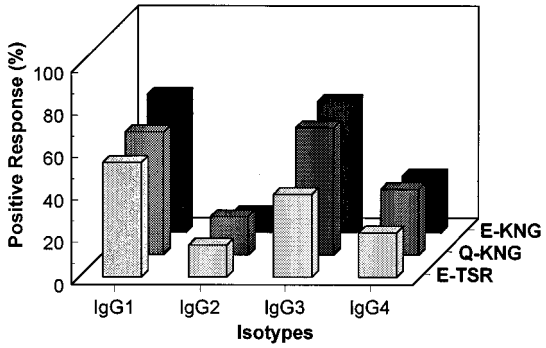

FIG. 3. Prevalence of IgG isotype responses to three variant $YMSP1_{19}$ antigens. Cutoff values are 0.046, 0.087, 0.046, and 0.048 for IgG1, IgG2, IgG3, and IgG4 (E-KNG), 0.072, 0.033, 0.073, and 0.028 for IgG1, IgG2, IgG3, and IgG4 (Q-KNG), and 0.03, 0.02, 0.042, and 0.014 for IgG1, IgG2, IgG3, and IgG4 (E-TSR), respectively. The highest dilution of the plasma samples which gave an OD above the cutoff value was expressed as titers. The plasma samples were scored as negative when the OD was less than the cutoff value at a 1:100 dilution.

SI values exceeded 10 for three individuals for the E-KNG construct, for four individuals for E-TSR, and for seven individuals for Q-KNG. These results indicate that the 19-kDa domain of the MSP-1 antigen contains natural T-cell determinants.

We also tested whether individuals not exposed to malaria (normal controls) could respond to the three $YMSP1_{19}$ fragments in a T-cell proliferative assay. The SI values for 15 normal individuals did not exceed 2.04 for E-KNG $YMSP1_{19}$, 2.29 for Q-KNG $YMSP1_{19}$, and 2.0 for E-TSR $YMSP1_{19}$, indicating that the yeast-expressed 19-kDa antigens mostly do not activate the population of T cells in malaria-naive individuals.

(ii) Comparison of proliferative responses of individuals to three variant $YMSP1_{19}$ antigens. In Fig. 2, the patterns of proliferative responses to three variant $YMSP1_{19}$ antigens are presented. We found that there is heterogeneity in the proliferative responses to the $YMSP1_{19}$ variants in different individuals. Among the 36 positive responders, 12 recognized all three variant $YMSP1_{19}$ antigens and 12 responded to two of three variants (E-KNG and Q-KNG, E-KNG and E-TSR, or Q-KNG and E-TSR). The remaining 12 individuals responded to only one of the three variant constructs. These findings suggest that the 19-kDa antigen may contain variant-specific, in addition to conserved, T epitopes.

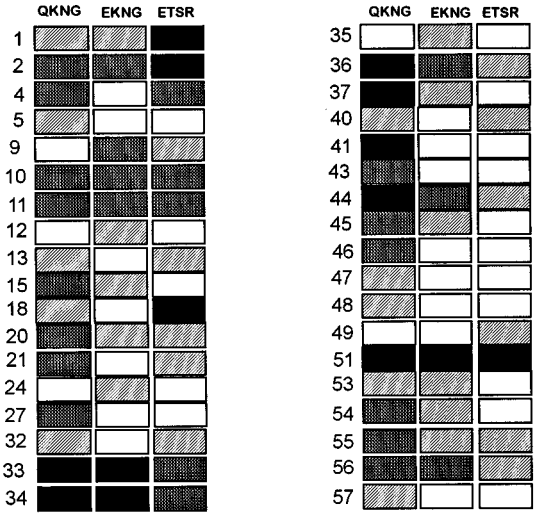

FIG. 2. Patterns of lymphoproliferative responses to three variant $YMSP1_{19}$ antigens in individuals. Data from individuals with positive responses are presented here. The numbers identify the individuals. The SI values for the proliferative responses have been stratified into four groups. ☐, negative; ▨, 2.5 to 5; ▩, 5 to 10; ■, >10.

IgG isotype response to $YMSP1_{19}$ antigens. (i) Prevalence and level of IgG isotype response. In a previous study, we showed that more than 75% of adults in western Kenya had positive total IgG antibody responses to the $YMSP1_{19}$ variant antigens and that maximum reactivity was found against the E-KNG antigen (25). In this study, we determined the profiles of different IgG isotype responses to the three variant $YMSP1_{19}$ antigens in 117 plasma samples from individuals aged 2 to 74 years. The prevalence of IgG1 antibody responses was 65% for E-KNG, 58% for Q-KNG, and 54% for E-TSR. The prevalence of IgG3 responses was 62% for E-KNG, 60% for Q-KNG, and 39% for E-TSR. Twenty-one to thirty-one percent of individuals had a low-level IgG4-positive response to the three variant $YMSP1_{19}$ antigens. Only 9.4 to 18% of individuals showed IgG2-positive responses with low titers to the three variant antigens (Fig. 3). The geometric means of IgG1 isotype titers for positive responders were 1/706, 1/862, and 1/891 for the E-KNG, E-TSR, and Q-KNG $YMSP1_{19}$ antigens, respectively. The geometric means of IgG3 antibody titers were 1/2,017, 1/2,123, and 1/1,852 for the E-KNG, E-TSR, and Q-KNG antigens, respectively (data not shown). These findings indicate that IgG1 and IgG3 antibodies are predominant over IgG2 and IgG4 antibodies in this population. The results also indicate that among the three variant $YMSP1_{19}$ antigens tested, IgG1 and IgG3 antibody responses to the E-KNG antigen are more common than responses to the other antigen forms in individuals from western Kenya. We also observed heterogeneity in the IgG1 and IgG3 isotype responses of individuals; e.g., some individuals showed a predominantly IgG1 response, some had a predominantly IgG3 response, and others exhibited both IgG1 and IgG3 responses (data not shown).

(ii) Age-dependent IgG1 and IgG3 response. As can be seen from Fig. 4A, the prevalence of IgG1 and IgG3 antibody responses to the E-KNG construct increased with age (for IgG1, $\chi^2 = 4.64$ and $P = 0.031$, and for IgG3, $\chi^2 = 4.61$ and $P = 0.032$). We also observed that total IgG1 and IgG3 antibody titers increased with age (Fig. 4B), with IgG3 titers showing more significant correlation with age than IgG1 titers ($r = 0.26$

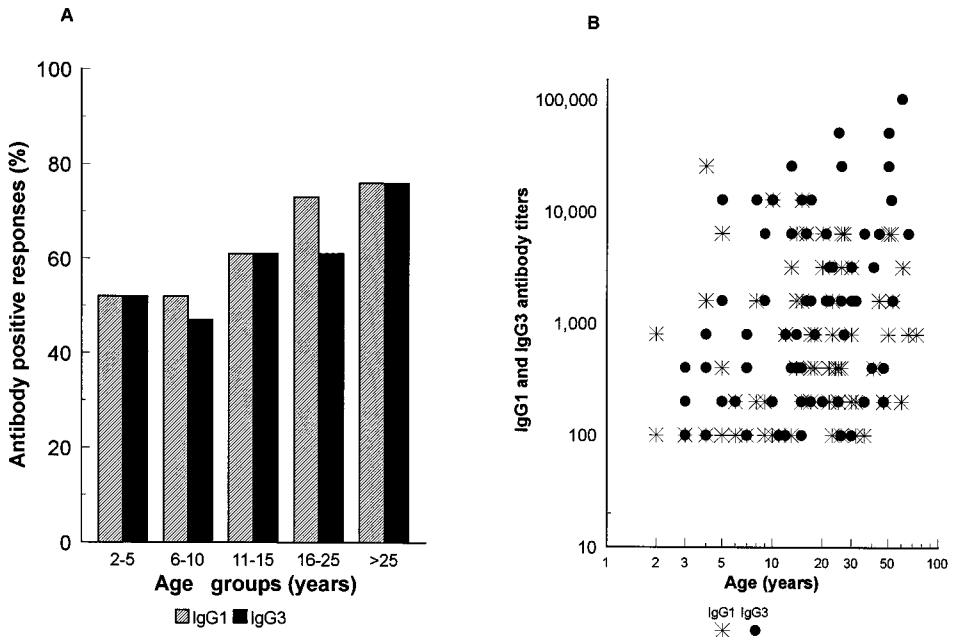

FIG. 4. Age-dependent IgG1 and IgG3 antibody responses. (A) Prevalence of age-dependent IgG1 and IgG3 antibody responses. Five groups were stratified according to age: 2 to 5 years ($n = 19$), 6 to 10 years ($n = 21$), 11 to 15 years ($n = 21$), 16 to 25 years ($n = 26$), and >25 years ($n = 30$). In chi-square tests for trend, $\chi^2 = 4.64$ and $P = 0.031$ for IgG1 and $\chi^2 = 4.61$ and $P = 0.032$ for IgG3. (B) Relationship between IgG1 and IgG3 antibody titers and age. The Spearman's rank correlation coefficients were $r = 0.26$ ($P = 0.025$) for IgG1 and $r = 0.34$ ($P = 0.0036$) for IgG3.

and $P = 0.025$ for IgG1 and $r = 0.34$ and $P = 0.036$ for IgG3). The same comparison was also done for antibody responses to the Q-KNG and E-TSR constructs, and similar results were obtained (data not shown).

(iii) Relationship between the antibody isotype response and age-dependent protection. In order to determine whether the IgG isotype antibody response to the E-KNG antigen correlates with protection against malaria, we examined the relationship between parasite density and age stratified by the antibody isotype response. Antibody response was divided into three groups: negative response, positive response with low antibody titer (≤400), and positive response with high antibody titer (>400). As can be seen from Fig. 5A, the effect of age on parasite density was associated with the IgG1 level. Younger individuals with high (>400) IgG1 antibody responses tended to have lower parasite densities compared with individuals of

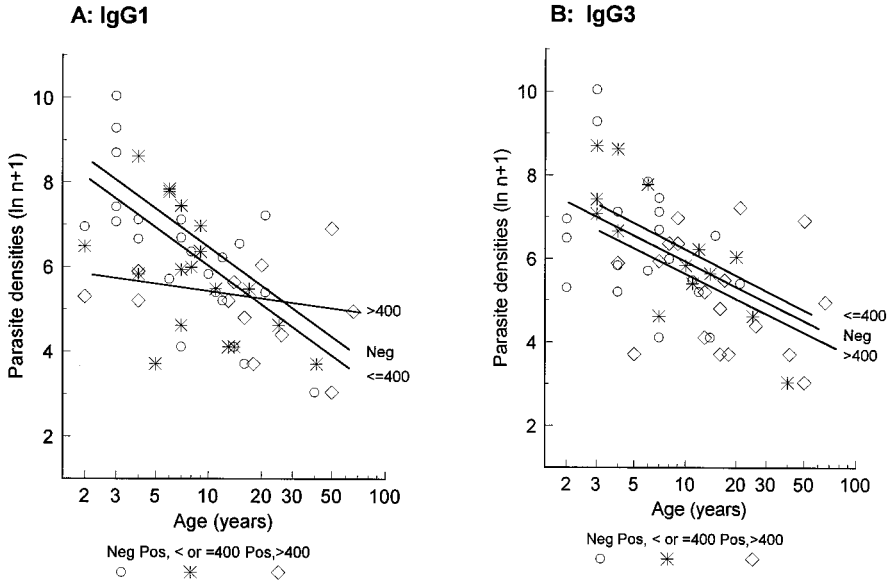

FIG. 5. Relationship between parasite density and age by IgG1 or IgG3 antibody response. (A) There was a significant difference between negative response and positive response groups with high titers ($F = 9.68$ and $P = 0.003$) but no difference between negative response and positive response groups with low antibody titers ($F = 0.78$ and $P = 0.38$). (B) There were no differences between negative response and positive response groups with low titers ($F = 0.36$ and $P = 0.55$) and between negative response and positive response groups with high antibody titers ($F = 0.21$ and $P = 0.65$).

TABLE 1. Association between the antibody titers for three $YMSP1_{19}$ antigens

Antigens	Correlation coefficient (*r*)	
	IgG1	IgG3
E-KNG and Q-KNG	0.93	0.89
E-KNG and E-TSR	0.86	0.66
Q-KNG and E-TSR	0.88	0.67

the same age with negative or low (≤400) responses. Low parasite density in the presence of high IgG1 is most evident for ages <5 years. These findings suggest that an IgG1 antibody response may be involved in protection against malaria. The results also imply that 19-kDa fragment-specific, IgG1 antibody-mediated protection develops at a young age. However, as can be seen from Fig. 5B, there is no difference in the outcomes for parasite density and age among the three groups in terms of IgG3 antibody response. A similar trend was consistently observed with antibody isotype responses to the Q-KNG and E-TSR construct antigens (data not shown).

Natural antibody cross-reactivities among three variant $YMSP1_{19}$ antigens. (i) Comparison of antibody recognition levels for three variant $YMSP1_{19}$ antigens. The association between the antibody titers of individual plasma samples and antibody recognition of the three $YMSP1_{19}$ variants was evaluated with the Pearson correlation coefficient (Table 1). We found similar levels of IgG3 antibody specific for both E-KNG and Q-KNG in most of the samples ($r = 0.89$). However, we observed differential recognition of IgG3 antibody for the E-KNG and E-TSR constructs and the Q-KNG and E-TSR constructs. Plasma samples from 12 individuals had high levels of IgG3 antibody responses to the E-KNG and Q-KNG constructs but no response to the E-TSR construct (data not shown). The *r* values for IgG3 levels for E-KNG and E-TSR and for Q-KNG and E-TSR are 0.66 and 0.67, respectively. In contrast to the differential recognition of the IgG3 antibodies, the differential recognition of the IgG1 antibodies for the three variant antigens was not profound. The *r* values of IgG1 titers for E-KNG and Q-KNG, E-KNG and E-TSR, and Q-KNG and E-TSR were 0.93, 0.86, and 0.88, respectively.

(ii) Determination of antibody cross-reactivity with the three $YMSP1_{19}$ antigens by competitive inhibition assay. In order to determine the cross-reactivities for antibody responses to the three variant antigens, a competitive inhibition assay was conducted. The IgG1 antibody responses to the E-KNG antigen in samples 22 and 52 were significantly inhibited by the variant Q-KNG and E-TSR antigens (Fig. 6A). Similarly, in plasma from individual 22, IgG3 antibody reactivity to the E-KNG antigen was completely inhibited by variant E-TSR and Q-KNG antigens (Fig. 6B). These data suggest that the IgG1 antibodies in samples 22 and 52 and the IgG3 antibodies in sample 22 cross-react with common immunogenic determinants within the three variant $YMSP1_{19}$ constructs. However, IgG3 antibodies in sample 14 had complete cross-reactivity for E-KNG and Q-KNG antigens (one amino acid substitution in the first EGF domain) but only partial cross-reactivity for the E-KNG (or Q-KNG) and E-TSR antigens (three amino acid substitutions in the second EGF domain) (Fig. 6B). This result suggests that sample 14 contains IgG3 antibodies that recognize the three dimorphic amino acid substitutions in the second EGF domain of the 19-kDa antigen.

DISCUSSION

In this study, we have examined the T-cell proliferative responses to three variant $YMSP1_{19}$ antigens in individuals older than 15 years. We found that 54% of individuals had positive proliferative responses to the Q-KNG antigen and that 37 and 35% of individuals responded to the E-KNG and E-TSR constructs, respectively. This result is consistent with our previous peptide-based T-cell proliferative study conducted in Kenya, which showed that three peptides from the MSP-1 19-kDa antigen stimulate lymphocyte proliferative responses in more than 30% of individuals tested (25). There is a question of whether peptides or recombinant polypeptides, disulfide-bonded polypeptides, or previously denatured polypeptides should be used for the study of T-cell responses to the 19-kDa region of MSP-1 (13). The major point of the concern is how this antigen is processed and recognized in vitro. Our previous and present studies were aimed at determining whether T-cell responses to the MSP-1 19-kDa antigen could be appropriately monitored with peptides or recombinant polypeptides in individuals naturally exposed to malaria or in vaccinated individuals. Comparison of these two studies has shown that recombinant polypeptides from the E-KNG and E-TSR $YMSP1_{19}$ antigens and three linear peptides from the E-TSR MSP-1 19-kDa antigen were equally recognized by Kenyan adults. However, so far as the recombinant polypeptide is concerned, we found in the present study that the Q-KNG $YMSP1_{19}$ antigen induced higher T-cell responses compared with those induced with the E-KNG and E-TSR $YMSP1_{19}$ antigens. It has been demonstrated that a single amino acid difference (E or Q) in the first EGF-like domain of the yeast-expressed recombinant 19-kDa fragment causes a significant change in disulfide bond-dependent conformation (17). A possible explanation for the differential prevalence of T-cell proliferative responses to the Q-KNG and E-KNG (or E-TSR) constructs in this study may be the different native three-dimensional structures of E-TSR, E-KNG, and Q-KNG, which could affect antigen processing and recognition in vitro. It will be of interest to further determine experimentally whether in vitro T-cell recognition is influenced by the different conformations of the MSP-1 19-kDa variant antigens with linear recombinant polypeptides. The data from this study also show that there was considerable heterogeneity in individual responses to the three variant forms of the 19-kDa antigen. Except for 12 positive responders who recognized three variant antigens, the remaining 24 individuals showed responses to only one or two of the three variant constructs. This result suggests that the 19-kDa antigen may also contain variant-specific T epitopes.

We also investigated the antibody isotype response to the $YMSP1_{19}$ antigen in individuals who participated in this study. Our data show that the IgG1 and IgG3 isotype responses were predominant over the IgG2 and IgG4 isotype responses. Both the IgG1 and IgG3 antibody responses increased with age. It was also found that a higher level of IgG1 antibody response correlated with lower parasite density among young age groups, suggesting that IgG1 antibody response may play a role in protection against malaria. Several recent cross-sectional and longitudinal studies have shown that total IgG antibodies against the C-terminal 42- and 19-kDa domains of MSP-1 correlate with protection against malaria (21–23). This investigation sought to delineate the relationship between IgG isotype antibody response to the 19-kDa antigen and protection against infection. Although the mechanisms of antibody-mediated in vivo protection are still not known, the observation that a high IgG1 isotype antibody level correlates with lower parasite density supports prior in vitro studies which indicated the

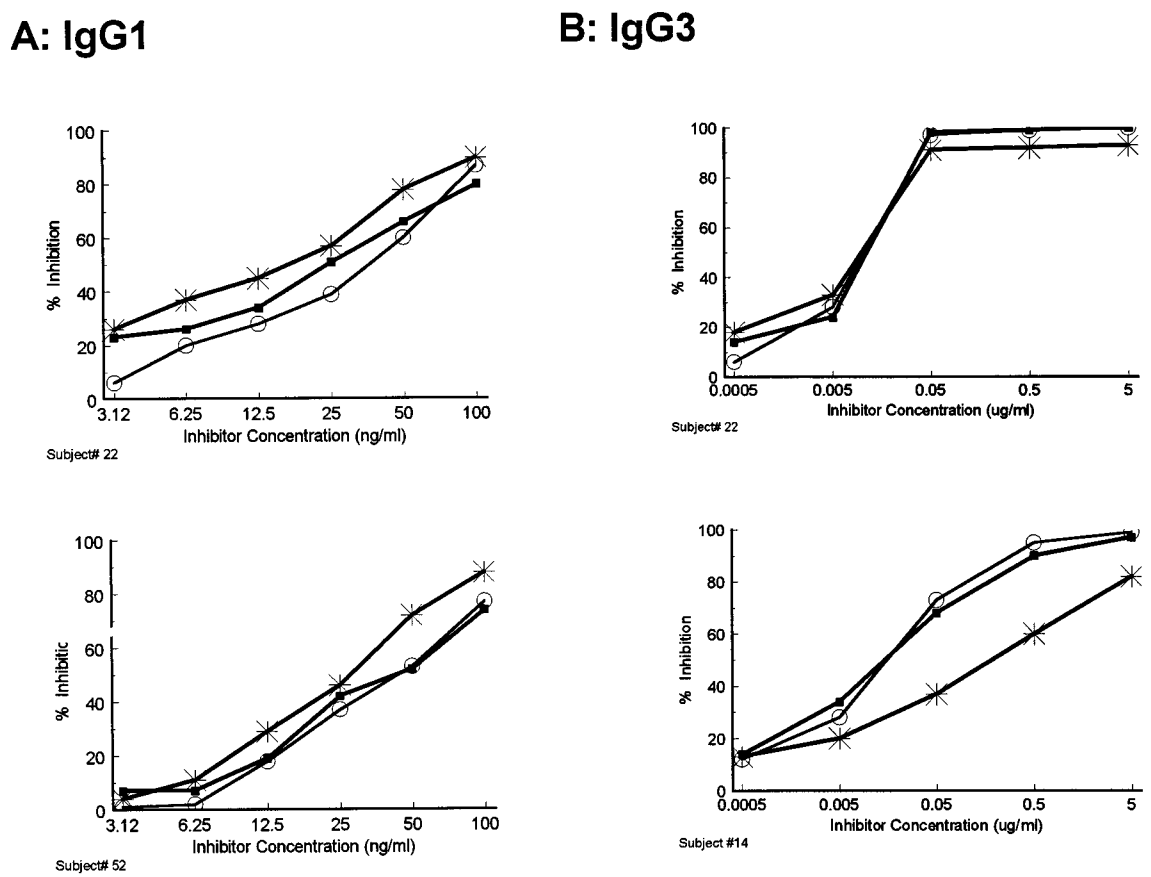

FIG. 6. Determination of antibody cross-reactivity with three $YMSP1_{19}$ antigens by competitive inhibition assay. (A) Two plasma samples (22 and 52) with equal avidities of IgG1 for three variant $YMSP1_{19}$ antigens were chosen to test the cross-reactivity of the IgG1 antibody. The plasma samples were diluted to obtain 50% IgG1 binding and then incubated with various concentrations of the E-KNG antigen (○), E-TSR antigen (∗), and Q-KNG antigen (■) inhibitors at 37°C for 1 h. Subsequently, the IgG1 antibody reactivity to the E-KNG antigen in the samples was determined by E-KNG-coated ELISA. (B) Two plasma samples (14 and 22) with equal avidities of IgG3 for three variant $YMSP1_{19}$ antigens were chosen to test the cross-reactivity of the IgG3 antibody. The plasma samples were diluted to get 50% IgG3 binding and then incubated with different concentrations of the E-KNG antigen (○), E-TSR antigen (∗), and Q-KNG antigen (■) inhibitors at 37°C for 1 h. The IgG3 antibody reactivity to the E-KNG antigen in the mixture was determined by E-KNG-coated ELISA.

existence of antibody isotype-dependent protection (4, 11). One such study revealed that antibody-dependent cellular inhibition of *P. falciparum*-infected erythrocytes was associated with cytophilic IgG1 and IgG3 isotypes (4). In another report, it was found that while all IgG isotypes of anti-*P. falciparum* antibodies bound to the surface of infected erythrocytes, only IgG1 and IgG3 antibodies were capable of mediating opsonizing phagocytosis, which is believed to be involved in protective immunity (11). It remains to be determined, however, whether the natural IgG1 antibodies against the 19-kDa antigen are invasion- and growth-inhibitory antibodies or if they mediate protection via antibody-dependent cytotoxic inhibition and opsonization mechanisms.

In contrast to our data, data from one recent study conducted in the town of Farafenni, a seasonally endemic area of Gambia, showed that antibody responses to the 19-kDa antigen were predominantly of the IgG1 isotype (9). The low IgG3 antibody response to the 19-kDa antigen in Gambians compared with that in Kenyans could be due to the different genetic backgrounds of these populations and/or different parasite inoculation rates in these study areas. It is also possible that the 19-kDa antigen-specific IgG3 antibody may have a short life. The differences between the IgG3 antibody responses reported in the present study and the aforementioned study (9) underline the importance of conducting similar immunoepidemiologic studies in other malarious areas where transmission intensities and human genetic backgrounds are different.

Development of protection against *P. falciparum* malaria is usually considered to be the cumulative effect of repeated exposure to parasites. Our epidemiological study in Asembo Bay, a holoendemic area of western Kenya, has revealed that while the prevalence of infection remains high among children 1 to 10 years old, there is a progressive ability to limit the occurrence of high-density infections and acute disease in children >5 years old. Most residents, by their teenage years, develop protective immunity, a state of premunition in which low parasite density prevails in the hosts without causing acute disease (17a). Numerous immunoepidemiologic studies have shown an increase in malaria-specific immune response with age. One question that has been raised pertains to the relationship and interaction between the age-dependent protection against malaria and the development of a malaria-specific immune response with age. The present study reveals that both IgG1 and IgG3 antibody responses to the 19-kDa antigen increase with age and that only higher IgG1 levels correlate with lower parasite density in younger age groups.

Although the C-terminal 19-kDa fragment of MSP-1 is highly conserved compared with other regions of MSP-1, amino acid substitutions have been found in laboratory and

field isolates of *P. falciparum* (15, 16, 19). One previous study has revealed that even a single amino acid difference (E or Q) in the first EGF-like domain of the 19-kDa antigen influences the binding of monoclonal antibodies to this domain (6). In the present study, we analyzed recognition of the three variant forms of the 19-kDa antigen (E-KNG, Q-KNG, and E-TSR) by naturally occurring antibodies. The results show that the natural polyclonal IgG1 and IgG3 antibodies react with conserved determinants in the three variants. However, the IgG3 antibodies in 12 plasma samples recognize three dimorphic amino acid substitutions in the second EGF domain (KNG and TSR) of the antigen. This result suggests that the fine specificity of IgG3 antibodies differentiates among variant-specific natural B-cell determinants in the second EGF domain of the antigen.

It became obvious that for the three variant $YMSP1_{19}$ antigens tested, IgG3 antibody responses to the E-KNG and Q-KNG antigens (62 and 60%, respectively) are more prevalent than those to the E-TSR antigen (39%) in western Kenyans. This result is consistent with our molecular epidemiologic study conducted in the same area in which we found that KNG-bearing parasites are predominant over TSR parasites (23a). We recognize that the profile of host immune responses may be dependent on the prevalence of the circulating strains of parasites, and for this reason we would like to suggest that similar immunoepidemiologic studies be conducted in other malarial regions.

In summary, this study shows that the 19-kDa domain of the *P. falciparum* MSP-1 antigen contains T-cell proliferative epitopes. IgG1 and IgG3 isotype responses to the $YMSP1_{19}$ antigen are predominant, with both IgG1 and IgG3 antibody responses increasing with age. We also found that a higher-level IgG1 antibody response is correlated with lower parasite density in younger age groups. The natural polyclonal IgG1 and IgG3 isotypes to the three variant 19-kDa constructs predominantly react with conserved determinants in the antigen, although IgG3 antibodies in 12 plasma samples recognized three dimorphic amino acid substitutions in the second EGF domain (KNG and TSR) of the antigen. For the three variant 19-kDa antigens tested, antibody responses to the E-KNG construct are most prevalent in Kenyans, as shown earlier. This investigation of the quality and quantity of the antibody isotype responses to the 19-kDa antigen in clinically immune and nonimmune individuals provides information on the characteristics of naturally acquired immunity against the 19-kDa domain of MSP-1 and the relationship between the specific immune response and protection against malaria. This information will be beneficial in the development and testing of a MSP-1-based malaria vaccine.

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