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Natural Antibody Responses against the Non-Repeat-Sequence-Based B-Cell Epitopes of the *Plasmodium falciparum* Circumsporozoite Protein

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Synthetic peptides and human serum or plasma samples from regions of Brazil, Papua New Guinea, and Kenya in which malaria is endemic were used to identify B-cell epitopes localized outside the repeat region of the circumsporozoite (CS) protein of the human malaria parasite *Plasmodium falciparum*. In agreement with recent observations, our results confirm the presence of two non-repeat-region-based B-cell epitopes of the CS protein. Of these two epitopes, only the region I epitope (KPKHKKLKQPGDGNP) was previously shown to be recognized by human sera. In this study, we show that human immune sera from malarious regions recognize another B-cell epitope, ENANANNAV, that resides carboxyl to the repeat region. The present study reveals that (i) the repeat-sequence (NANP)-based B-cell epitope of the CS protein is an immunogenic but not immunodominant epitope; (ii) the natural expression of antibody responses to the two non-repeat-region-based B-cell epitopes of the CS protein varies in different populations in which malaria is endemic; (iii) although the host immune responses to the non-repeat-region-based B-cell epitopes increase as a function of host age, this increase is not statistically significant for the region I epitope but is significant for the other epitope; and (iv) the Th1R T-cell site but not the Th2R or Th3R T-cell site induces an antibody response in the human host. This study confirms the immunogenic potential of non-repeat-region-based B-cell epitopes and suggests that antibody pressures may also contribute to the maintenance of the antigenic diversity of the CS protein.

Characterizing parasite antigens and understanding host immune responses against their determinants under conditions of natural exposure to parasites are essential in malaria vaccine development (19). Several *Plasmodium falciparum* vaccine candidate antigens have been characterized, and vaccine formulations containing B- and T-cell epitopes have been prepared (2, 7, 13, 20). Among *P. falciparum* antigens, the circumsporozoite (CS) protein is the most extensively characterized. The genes encoding the CS protein have been cloned and sequenced for rodent, monkey, chimpanzee, and human malaria parasites. These CS proteins generally contain a central repetitive B-cell epitope flanked by genus-conserved regions, referred to as region I and region II. In human malaria parasite *P. falciparum*, the central repeat region of the CS protein is composed of NANP (major) and NVDP (minor) repeat sequences (6). The non-repeat region of the CS protein contains polymorphic T-cell determinants and putative anchor and signal sequences. The identified T-cell sites of the CS protein are (i) Th1R, a T-cell proliferation site that overlaps the putative hepatocyte binding site (N-1 site); (ii) Th2R, a helper T-cell site that resides carboxyl to the repeat region; and (iii) Th3R, a T-cell proliferation site that contains the only identified $CD8^+$ cytotoxic T-cell (CTL) epitope of the CS protein (1, 11, 15).

Seroepidemiologic investigations designed to assess natural host immune responses to CS proteins have revealed that the humoral immune response of the host against the CS protein repeat-region-based epitope increases in an age-dependent manner (4, 5, 10, 14, 17, 18). Research to develop a CS protein-based sporozoite vaccine has been based on in vitro experiments with CS protein repeat-sequence-specific monoclonal antibodies (MAbs) that demonstrate the protective nature of the CS protein (28). However, although in vitro experiments have shown evidence of strong inhibitory effects of anti-CS repeat sequence antibodies, the outcome of in vivo testing of CS protein repeat sequence constructs in human vaccine trials has not been promising (2, 8, 13). The failure of these CS vaccine formulations could be accounted for by the inability of the vaccines to induce a protective antibody response, the inability of the vaccines to involve the cell-mediated arm of host immunity, and/or a lack of other epitopes in the vaccine constructs that may be essential in conferring optimal levels of immunity in vivo.

Several laboratories are directing efforts towards addressing each of these issues to lead to a better understanding of the nature of protective immunity against the sporozoite stage of the malaria parasite. Recent investigations with CS peptides, MAbs, and serum samples from regions in which malaria is endemic have led to the identification of three additional B-cell epitopes that reside outside the repeat region of the CS protein. One epitope (KPKHKKLK QPGDGNP), recognized by sera from individuals in an area of Gabon in which malaria is endemic, resides amino terminal to the repeat region that overlaps genus-conserved region I. The other two B-cell epitopes (PNDPNRNVD and ENANANNAV), identified by use of mouse MAbs, reside carboxyl terminal to the repeat region (3, 9, 26). It is not known

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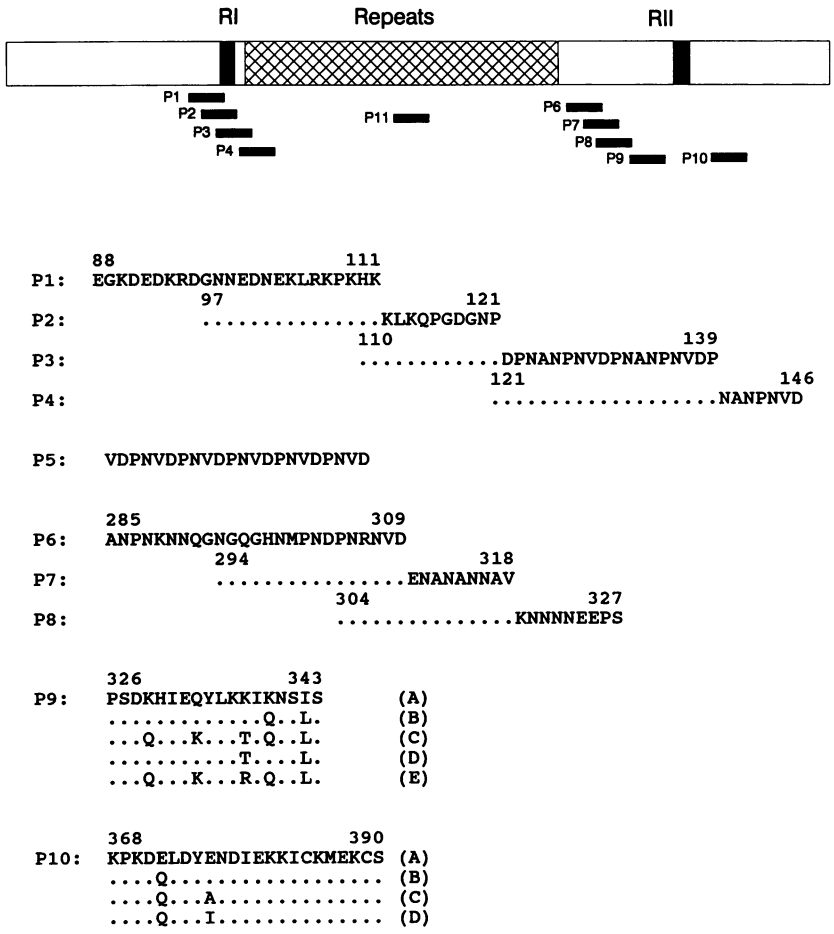

FIG. 1. Locations and sequences of 10 peptides of the *P. falciparum* CS protein used in this study. Peptides P1 and P2, P2 and P3, P3 and P4, P6 and P7, and P7 and P8 overlap by 12 to 18 amino acids. Peptide P1 contains the amino-terminal sequence of the Th1R T-cell epitope; peptide P2 is complementary to the Th1R T-cell epitope and conserved region I; peptide P3 contains region I and two copies of NANP plus NVDP; peptide P4 contains three copies of NANP plus NVDP; and the recently identified non-repeat-region-based B-cell epitopes are located in overlapping domains of peptides P6, P7, and P8. The sequence represented by peptide P5 (five copies of the minor repeat NVDP) is not a natural sequence in the CS protein of *P. falciparum*. Peptides P9 (A to E) and P10 (A to D) represent known polymorphic T-cell immunodominant determinants, Th2R and Th3R, respectively.

whether the PNDPNRNVD and ENANANNAV epitopes are immunogenic in humans naturally exposed to malaria. Similar studies conducted with *P. vivax* and *P. knowlesi* CS proteins have revealed the presence of B-cell epitopes outside the repeat region of the protein (22, 23, 27).

Despite several seroepidemiologic studies on the role of NANP repeat determinants in protection, the identification or significance of B-cell epitopes in the non-repeat regions of the CS protein, under conditions of natural infection, is not well understood. This study was designed to determine the natural host response to the non-repeat determinants of the CS protein and to address three interrelated issues that are important in assessing the development of natural immunity to the CS protein: (i) determine the natural humoral immune response to the non-repeat regions of the CS protein, including two B-cell epitopes localized in the C-terminal region and identified on the basis of the reactivity of the mouse MAbs; (ii) evaluate differences in host immune responses to the non-repeat and repeat CS protein determinants in three geographically different regions in which malaria is endemic; and (iii) determine whether the humoral immune response to these non-repeat-region-based B-cell epitopes increases as a function of the age of the host. To address each of these issues, we made synthetic peptides spanning the CS protein to screen for the presence of specific antibodies in human serum or plasma samples collected from regions of Brazil, Papua New Guinea (PNG), and Kenya in which malaria is endemic.

MATERIALS AND METHODS

Peptides. The amino acid sequences of the peptides used in this study are shown in Fig. 1. Peptides P1 to P8 were synthesized at the Biotechnology Core Facility of the National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga., as described previously (25). The amino acid sequences of these peptides, except for P5, were based on the CS protein sequence of the *P. falciparum* 7G8 clone (6). These peptides were further purified by high-pressure liquid chromatography. The sequence of peptide P5 is not a natural sequence of the CS protein, so P5 was used as a control peptide. Peptides P9 and P10 represent polymorphic Th2R (helper T-cell) and Th3R (CTL epitope) determinants of the CS protein, respectively (16, 23a, 24). Some of

the peptides corresponding to the polymorphic Th2R and Th3R regions were kindly provided by Vidal de La Cruz, MedImmune, Gaithersburg, Md. P11 is a recombinant protein, designated R32LR, which contains 30 NANP tetrapeptides and 2 NVDP repeats and was a generous gift from Smith Kline Beecham Pharmaceuticals, King of Prussia, Pa.

Human serum or plasma collection. Seventy-nine plasma samples were collected in Paragominos and Jacunda, Brazil, areas with a relatively low level of endemicity of malaria, during May and June of 1990 and 1991. During July 1990, 116 plasma samples were obtained from Madang Province, a region in PNG in which malaria is holoendemic. In September 1991, serum samples were collected from 129 individuals in Nyansa Province, an area in western Kenya in which malaria is highly endemic. The venous blood, drawn in Vacutainer tubes (Becton Dickinson, Rutherford, N.J.), was allowed to clot, and the serum was separated after overnight incubation at 4°C. The plasma was separated from the venous blood, drawn in Vacutainer tubes containing heparin (Becton Dickinson), by spinning at 2,000 rpm for 10 min. The plasma and serum samples were stored at −70°C until use. Forty-nine normal adult serum samples from the Centers for Disease Control Blood Bank were used as negative controls. All the serum samples were collected without any preference for age, race, or sex. The serum or plasma samples were individually diluted to 1:100 for quantitation of antibody to the peptides in the Falcon assay screening test–enzyme-linked immunosorbent assay (FAST-ELISA).

FAST-ELISA. Levels of antibodies against the CS peptides in the serum or plasma samples were determined by the FAST-ELISA as described elsewhere (12). In brief, the individual peptides were used to coat plastic beads by incubation of the beads with 100 μl of individual peptides (10 μg/ml) in phosphate-buffered saline (PBS) (0.01 M; pH 7.4) for 2 h. After being incubated, the beads were rinsed with PBS containing 0.5% Tween 20 (PBS-T) and distilled water, air dried, and used immediately or stored in desiccant at room temperature until use. The serum or plasma samples were diluted to 1:100 in PBS-T containing 5% nonfat lyophilized milk (PBS-T-M). Antigen-coated beads were incubated with 100 μl of prediluted test samples for 5 min, washed with PBS-T and then with distilled water, and incubated with peroxidase-conjugated goat anti-human immunoglobulin G (Fisher Scientific, Pittsburgh, Pa.) at a 1:500 dilution in PBS-T-M for 5 min. After a wash, the beads were incubated with 150 μl of 3,3′,5,5′-tetramethylbenzidine (Microwell Peroxidase Substrate System; KPL Laboratories, Gaithersburg, Md.) solution for 5 min for color development. The A_{650} was read with a Titertek Multiskan reader (Flow Laboratories, McLean, Va.). All samples were individually tested in duplicate against each peptide. A positive reaction of samples from areas in which malaria was endemic was defined as an optical density (OD) exceeding the mean OD plus 3 standard deviations for the 49 normal control serum samples from Americans without a history of exposure to malaria.

Competitive peptide inhibition assay. A competitive inhibition assay was used to map the antibody binding region in a given peptide. The rationale for this assay is that any peptide that shares an epitope with another test peptide will block the reactivity of an antibody in serum or plasma samples to the test peptide. To do this experiment, we first established a standard curve for individual sera or a pool of sera and then chose the 50% binding point dilution to perform the competition experiment. The diluted serum or plasma samples were incubated with different concentrations of competing peptides for 1 h at 37°C before being allowed to react with test antigen-coated beads in the FAST-ELISA as described above.

TABLE 1. Prevalence of specific antibodies in human serum or plasma samples from three areas in which malaria is endemic

Peptide	No. positive/no. tested (% positive) for samples from[a]:		
	Brazil	PNG	Kenya
P1	8/79 (10.1)	23/116 (19.8)	16/129 (12.4)
P2	23/79 (29.1)	43/116 (37.1)	9/129 (6.9)[b]
P3	18/79 (22.8)	31/116 (26.7)	44/129 (34.1)
P4	6/79 (7.6)	9/116 (7.8)	36/129 (27.9)[c]
P5	2/79 (2.5)	10/116 (8.6)	19/129 (14.7)
P6	7/79 (8.9)	7/116 (6.0)	15/129 (11.6)
P7	25/79 (31.6)	36/116 (31)	19/129 (14.7)[d]
P8	16/79 (20.3)	29/116 (25)	16/129 (12.4)[e]
P9[f]	2/38 (5.2)	3/71 (4.2)	2/129 (1.6)
P10[f]	2/38 (5.2)	6/71 (8.5)	10/129 (7.8)
P11	23/79 (29.1)	38/116 (32.8)	68/129 (52.7)[g]

[a] When the χ^2 test or the Fisher exact test was used to compare the rates of positive antibody responses in serum or plasma samples from PNG and Brazil, all P values were >0.05. When the frequencies of positive responses for Kenyan samples were compared with those for samples from PNG and Brazil, significant differences were found.

[b] $\chi^2 = 26.8$; $P = 0.0000015$.

[c] $\chi^2 = 23.9$; $P = 0.0000064$.

[d] $\chi^2 = 11.45$; $P = 0.0033$.

[e] $\chi^2 = 6.48$; $P = 0.039$.

[f] On the basis of variants of the Th2R and Th3R T-cell epitopes of the *P. falciparum* CS protein sequences found in Brazil, PNG, and Kenya (23, 23a) different polymorphic peptides, P9 and P10, were used to detect antibody responses. Peptides P9-A, P9-B, P10-B, and P10-C were used to detect antibody reactivity to Th2R and Th3R T-cell epitopes in samples from PNG and Brazil; antibody reactivity to the Th2R and Th3R T-cell epitopes in samples from Kenya was determined by use of peptides P9-C, P9-D, P9-E, P10-A, P10-B, P10-C, and P10-D.

[g] $\chi^2 = 15.14$; $P = 0.0051$.

Statistical analysis of results. The χ^2 test or the Fisher exact test was used to test the statistical significance of positive frequencies in different population groups and the proportional prevalence of antibody reactivities in different age groups. P values of <0.05 were considered significant.

RESULTS

Non-repeat-sequence-based B-cell determinants of the CS protein of *P. falciparum*. The 324 serum or plasma samples from individuals living in the three regions in which malaria is endemic (Brazil, PNG, and Kenya) were tested for the presence of antibodies against various peptides spanning both repeat and non-repeat regions of the CS protein. Control sera collected from 49 healthy individuals were also concurrently tested against these peptides. The mean OD and standard deviation for the control sera against test peptides were 0.089 (range, 0.065 to 0.115) and 0.079 (range, 0.031 to 0.141), respectively. The mean OD plus 3 standard deviations was used as the cutoff value to score for a positive reaction.

Antibodies against the *P. falciparum* CS repeat sequence NANP were determined by testing the reactivity of serum or plasma antibodies with construct P11; Kenyan sera showed the highest antibody prevalence (52.7% positive); these were followed by PNG (32.8%) and Brazilian (29.1%) samples (Table 1 and Fig. 2). Levels of antibodies against the CS repeat sequence increased as a function of host age (Fig. 3).

When synthetic peptides that spanned regions outside the repeat region of the CS protein were used as capture

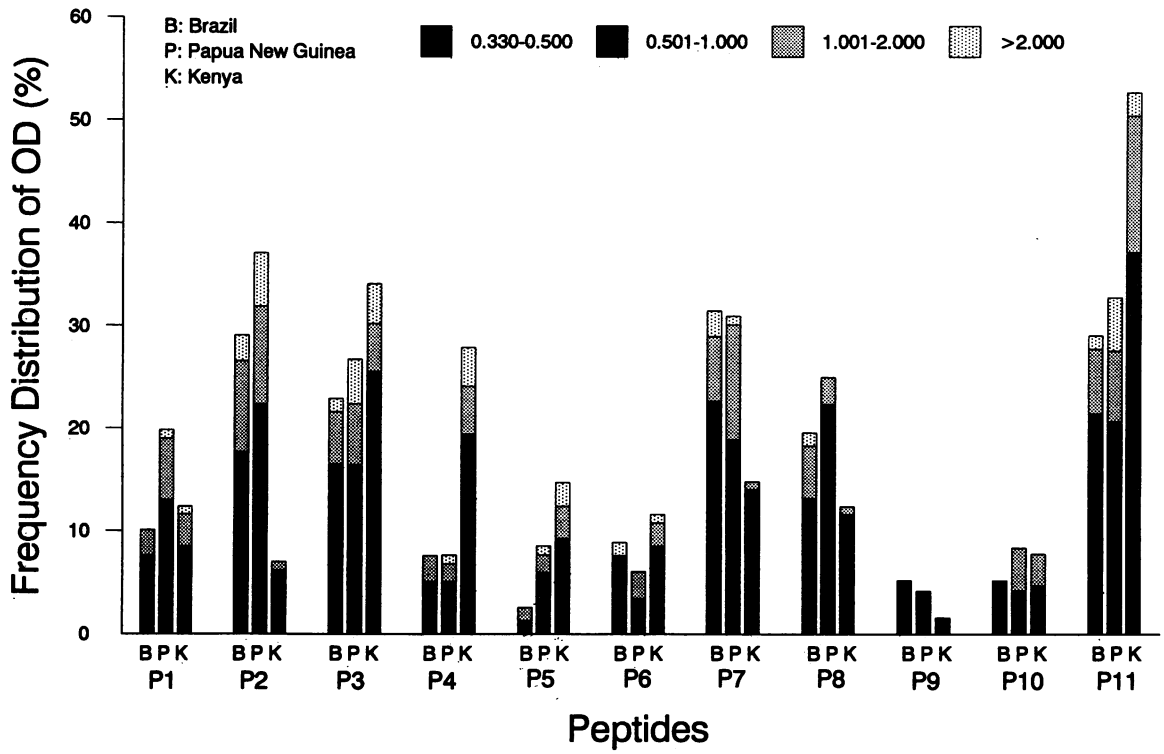

FIG. 2. Frequency distribution of ODs for antibody-positive samples from three areas in which malaria is endemic. PNG, Brazilian, and Kenyan serum or plasma samples positive for antibodies to different peptides were separated into four groups based on their ODs: 0.330 to 0.500, 0.501 to 1.000, 1.001 to 2.000, and >2.000, respectively. The frequency distribution of ODs is a representation of percent seropositive cases.

antigens in the FAST-ELISA, we found antibodies that reacted to the epitopes outside the repeat region of the CS protein. In the PNG and Brazilian samples, the highest seropositivity was found against peptides P2 and P3, representing the amino-terminal end of the CS protein (Fig. 2). In contrast, the Kenyan samples showed a different pattern of host antibody response to these peptides; while very low levels of antibodies to peptide P2 were detected, high seropositivity against peptides P3 and P4 was observed (Fig. 2). The rates of positive antibody responses to peptide P2 in plasma samples from Brazil and PNG were 29.1 and 37.1%, respectively, compared with only 6.9% of Kenyan serum samples that recognized this determinant. The antibody reactivity to peptide P4, which contains three copies each of NANP and NVDP repeats, indicates that nearly 28% of Kenyan samples in this study had antibody that recognized the P4 peptide, while fewer than 10% of Brazilian and PNG samples scored positive. Peptide P1 was recognized by 10.1% of Brazilian, nearly 20% of PNG, and 12.4% of Kenyan samples. However, the highest seropositivity in all three population groups was found against peptide P3. Since the sequence of peptide P3 overlaps the sequences of both peptide P2 and peptide P4 (Fig. 1), a simple explanation could be that the reactivity to peptide P3 represents binding to overlapping immunogenic sequences of P2 (HKKLK QPGDGNP) or P4 (two copies of NANPNVDP).

For determination of which antigenic site of peptide P3 accounts for the antibody activity in different population groups, a competitive inhibition experiment was carried out (Fig. 4). Because PNG and Brazilian plasma samples had similar patterns of immune responses to the CS peptides, we pooled the plasma samples from these two study areas for an epitope mapping study. The antibody reactivity of the plasma samples from PNG and Brazil to peptide P2 was inhibited by preincubation of the samples with competing peptide P3 but not peptides P1 and P4 (Fig. 4A). Because peptides P2 and P4 each share 12 carboxyl-terminal amino acid (aa) residues and 18 amino-terminal aa residues, respectively, with peptide P3, the antigenic site in peptide P2 should be localized near the overlapping sequence of peptides P2 and P3, which is HKKLKQPGDGNP. This antigenic sequence is identical to the recently identified B-cell determinant CKHKKLKQPGDG (3, 7) and hereafter is referred to as the region I B-cell epitope. Since PNG and Brazilian samples showed minimal reactivity to peptide P4, as shown in Fig. 2, and 77.6% of anti-P3-positive samples had antibody to peptide P2 (data not shown), it is apparent that the reactivity to peptide P3 could be attributed to the region I B-cell epitope. These experiments suggest that the region I B-cell epitope is highly immunogenic in PNG and Brazilian populations. Figure 4B shows that the anti-P4 reactivity of the Kenyan sera was blocked by competing peptide P3 but not peptides P1 and P2, a result suggesting that the competing antigenic site in peptide P3 must be the NANPNVDP repeat region. Since Kenyan sera did not show significant reactivity to peptide P2 (Fig. 2) and 80.9% of the Kenyan anti-P3-positive samples also recognized peptide P4 (data not shown), their anti-P3 reactivity should represent binding to the NANPNVDP repeat region.

Among the peptides representing the carboxyl terminus of the CS protein, the highest rate of reactivity was found with peptide P7, followed by peptide P8. A comparison at the

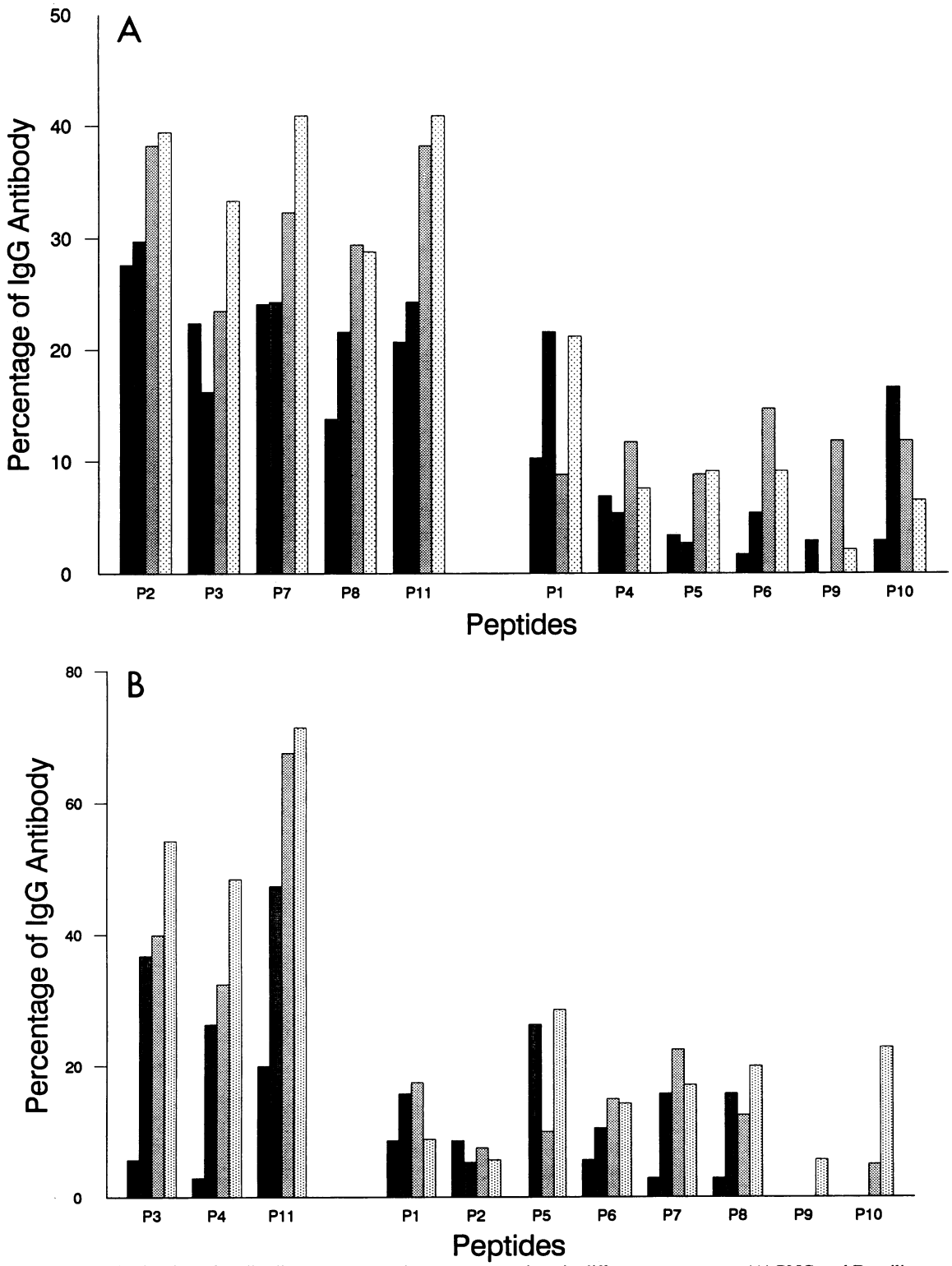

FIG. 3. Frequency distribution of antibodies to repeat and non-repeat regions in different age groups. (A) PNG and Brazilian populations. Because antibody responses to most of the peptides in malaria-infected individuals from Brazil and PNG were similar, the data for these two areas were pooled and the positive responders for each peptide were divided into four age groups: 0 to 5 years (■) (n = 58), 6 to 10 years (■) (n = 37), 11 to 20 years (▨) (n = 34), and >20 years (□) (n = 66). The χ^2 test for an age linear trend showed that the χ^2 values for peptides P2, P3, P7, P8, and P11 (NANP repeats) were 2.32, 2.51, 4.63, 4.30, and 7.03; the P values were 0.127, 0.113, 0.031, 0.038, and 0.0080, respectively. (B) Kenyan population. The positive responders among Kenyans were also classified into four age groups: 0 to 5 years (■) (n = 35), 6 to 10 years (■) (n = 19), 11 to 20 years (▨) (n = 40), and >20 years (□) (n = 35). The χ^2 values for peptides P3, P4, and P11 were 17.96, 18.08, and 21.56; the P values were 0.00002, 0.00002, and <0.00001, respectively. IgG, immunoglobulin G.

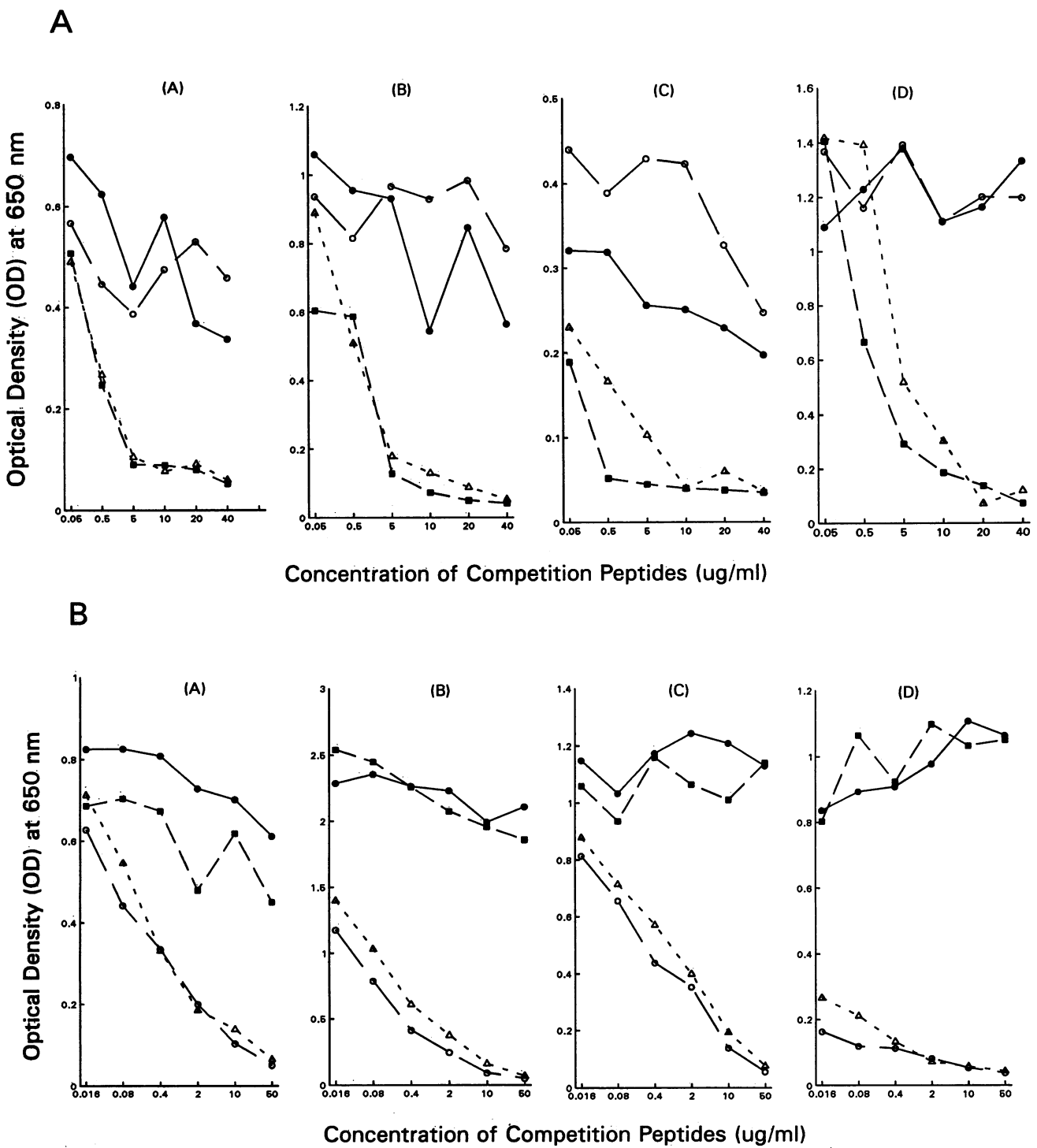

FIG. 4. Mapping the fine antigenic sites in overlapping peptides by competitive inhibition. Since peptides P1 and P2, P2 and P3, P3 and P4, P6 and P7, and P7 and P8 overlap, the antibody binding targets in peptides P2, P3, and P4, and P7 and P8 recognized by Brazilian, PNG, or Kenyan samples were determined by competitive inhibition. (A) Determination of the antibody binding site in peptide P2. Of the four test samples, A, B, and C represent plasma samples from PNG and D represents a plasma sample from Brazil. The samples were diluted to 1:140, 1:150, 1:140, and 1:270, respectively, to achieve 50% binding for each sample and consecutively incubated with various concentrations of peptide P1 (●), P2 (■; positive control), P3 (△), or P4 (○) at 37°C for 1 h. Subsequently, the antibody reactivity to peptide P2 in the four samples was detected by a FAST-ELISA. (B) Determination of the antibody binding site in peptide P4. Four pooled Kenyan serum samples (A, B, C, and D) were diluted to 1:100, 1:170, 1:150, and 1:120, respectively, to achieve 50% binding for each sample and then incubated with peptide P1 (●), P2 (■), P3 (△), or P4 (○; positive control). Following incubation, the serum samples were tested in a FAST-ELISA with peptide P4-coated pins. (C) Determination of the antibody binding site in peptide P7. Plasma samples from PNG (A, B, and C) and Brazil (D) were diluted to 1:130, 1:150, 1:250, and 1:130, respectively, to achieve 50% binding for each sample, preincubated with peptide P6 (●), P7 (■; positive control), or P8 (△), and then tested in a FAST-ELISA with peptide P7-coated pins. When peptide P5 (40 μg/ml) was used as a competing peptide (negative control), no inhibition was invariably found in all the tests.

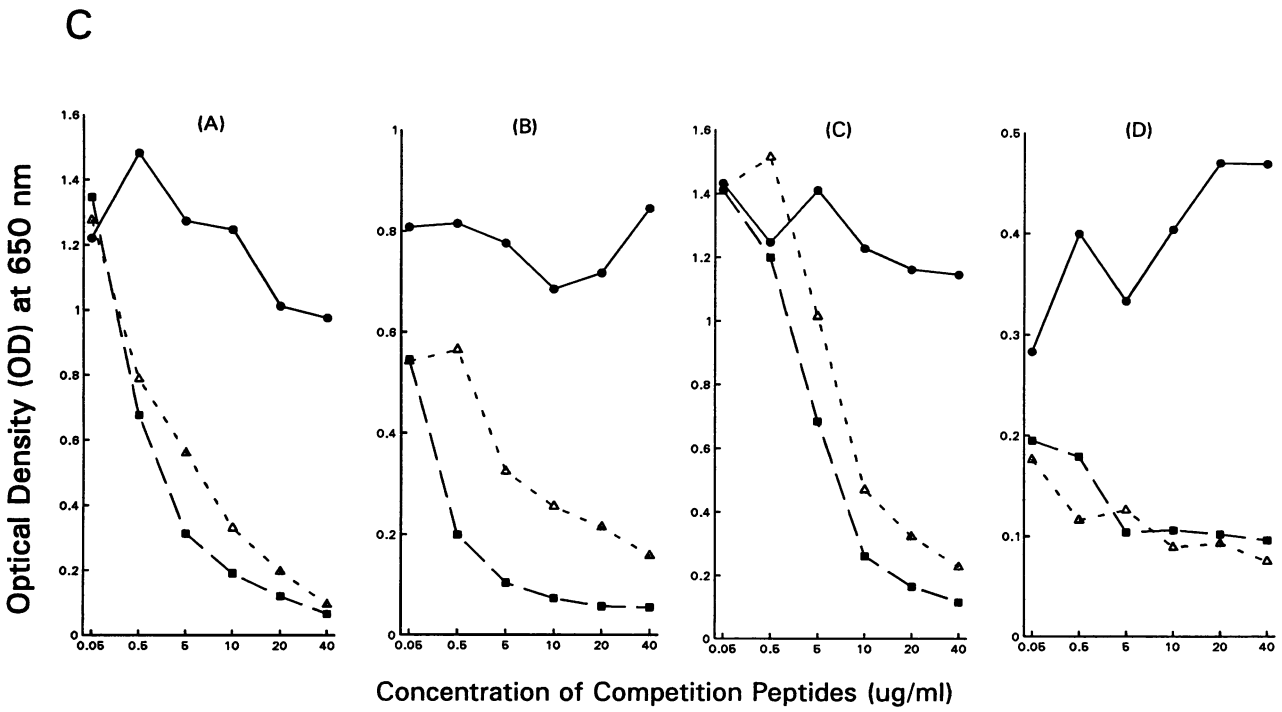

FIG. 4—*Continued.*

population level showed that reactivity to peptides P7 and P8 was lower among Kenyans (14.7 and 12.4%, respectively) than among Brazilians (31.6 and 20.3%) and Papuans (31 and 25%). Peptide P7 contains sequences representing at least two known B-cell epitopes, PNDPNRNVD (aa 301 to 309) and ENANANNAV (aa 310 to 318) (26). By comparing the overlapping sequences between peptides P7 (aa 294 to 318) and P6 (including aa 301 to 309 but not aa 310 to 318) or P8 (containing aa 304 to 318), we predicted that either the sequence PNDPNRNVD or the sequence ENANANNAV could be the likely target for antibody binding to peptide P7. To confirm this prediction, we conducted a competitive inhibition assay with peptides P6 and P8 for anti-P7 reactivity in PNG and Brazilian plasma samples. The antibody reactivity to peptide P7 in PNG and Brazilian samples was significantly blocked by peptide P8 but not peptide P6, which contains the PNDPNRNVD sequence (Fig. 4C). These results suggest that the sequence PNDPNRNVD in peptide P7 is not a component of the antigenic site recognized by these plasma samples. Since peptides P7 and P8 both contain the sequence ENANANNAV and 66.7% of anti-P8-positive samples from Papuans and Brazilians had antibody to peptide P7 (data not shown), their reactivity to peptide P8 could reflect binding to the sequence ENANANNAV.

Host age-dependent natural immunity to the non-repeat-sequence-based B-cell immunodominant determinants. Earlier studies and our results showed that the development of antibodies to NANP repeats is age dependent (4, 5, 10, 14, 17, 18). To determine whether a similar correlation exists for the non-repeat regions of the CS protein, we stratified antibody-positive rates by age. Since Brazilian and PNG populations exhibited similar trends in the prevalence of antibodies to most of the peptides (Table 1 and Fig. 2), their data were combined for this analysis (Fig. 3A). As shown in Fig. 3A, the percentage of responders for peptides P2 and P3 (representing epitope HKKLKQPGDGNP) and P7 and P8 (representing epitope ENANANNAV) did show an increase as a function of age. However, this difference was statistically significant for peptides P7 and P8 ($P = 0.031$ for peptide P7 and $P = 0.038$ for peptide P8) but not for peptides P2 and P3 ($P = 0.127$ for peptide P2 and $P = 0.113$ for peptide P3), as determined by the χ^2 test. As shown in other investigations (4, 5, 10, 14, 17, 18), antibody responses against repeat sequence NANP (recombinant protein P11) increased significantly as a function of age ($P = 0.008$). The data for the Kenyan population are given in Fig. 3B. The Kenyan population showed major reactivity to repeat sequence NANP, as represented by peptides P3 and P4 and recombinant protein P11, and this response increased significantly in an age-dependent manner ($P = 0.00002$ for peptide P3, $P = 0.0002$ for peptide P4, and $P < 0.00001$ for recombinant protein P11) (Fig. 3B). In contrast, such an age-dependent relationship did not exist for the other peptides (Fig. 3).

Natural humoral responses to the CS protein T-cell determinants. The major T-cell determinants of the *P. falciparum* CS protein are located in the polymorphic non-repeat regions of the protein. The sequence from aa 103 to 116, referred to as Th1R and partially overlapping the region I sequence, induces T-cell proliferation; the sequence from aa 326 to 343, referred to as Th2R, induces helper T-cell activity; and the sequence from aa 368 to 390, referred to as Th3R, induces T-cell proliferation and contains a CTL determinant of this vaccine antigen (1, 11, 15). In our experiments, peptide P2 spanned Th1R, peptide P9 represented five polymorphic Th2R determinants, and peptide P10 represented four polymorphic peptides complementary to the Th3R region of the CS protein. On the basis of the results of these experiments (Table 1 and Fig. 2), we conclude that only the Th1R T-cell determinant induces a host B-cell response in PNG and Brazilian populations. FAST-ELISA with peptides P9 and P10, which contain Th2R and Th3R T-cell determinants, respectively, did not

reveal any appreciable level of reactivity in tests with Brazilian, PNG, and Kenyan serum or plasma samples.

DISCUSSION

Although the CS protein is the most immunologically characterized protein of the malaria parasite, the goal of using this protein in an effective vaccine against malaria has not yet been realized. Until recently, all efforts towards sporozoite vaccine development were based on constructs involving the CS repeat sequences (2, 7, 8, 13). We initiated a study to identify other B-cell epitopes of the CS protein that are immunogenic under natural conditions of parasite exposure and could be used in a sporozoite vaccine formulation. The study was designed to measure in naturally exposed individuals total anti-CS protein immunoglobulin G antibody against the non-repeat and repeat regions of the CS protein from different regions in which malaria is endemic and from different age groups.

In agreement with other investigators (3, 8, 26), we found that two non-repeat-region-based B-cell epitopes in the CS protein of *P. falciparum* were recognized by serum or plasma samples from individuals naturally exposed to malaria. We observed that individuals from three different areas (Brazil, PNG, and Kenya) differed in terms of their ability to respond to these two antigenic determinants. The major difference among the three study populations was the failure of the Kenyan population to respond to the region I B-cell epitope, as measured by poor antibody reactivity to peptide P2. Antibodies against the region I epitope have been shown to block sporozoite invasion into human hepatoma cells in vitro (1, 9). Moreover, serum samples from individuals naturally infected with *P. vivax* contained antibodies to region I, and serum antibodies in monkeys and rabbits vaccinated with *P. knowlesi* sporozoites recognized region I (22, 23, 27). Taken together, these results reveal a potential role for this genus-conserved region I B-cell epitope in sporozoite vaccine formulation. It is intriguing, however, that serum samples from Kenya failed to recognize the region I B-cell epitope. Perhaps the failure of Kenyan serum antibodies to recognize the region I B-cell epitope is due to differences in the histocompatibility leukocyte antigen makeup of the Kenyan, PNG, and Brazilian populations and/or to natural variations in the population of *P. falciparum* malaria parasites from Kenya.

The Kenyan samples also differed from the PNG and Brazilian samples in their response to the epitopes within peptides P7 and P8. A lower percentage of Kenyan samples than of Brazilian and PNG samples were positive for these two peptides. Peptide P7 contains two B-cell epitopes that were shown to be recognized by mouse MAbs (26). It became apparent from competitive inhibition experiments that PNG and Brazilian sera recognized only one of these epitopes (ENANANNAV), the sequence of which also overlapped the sequence of peptide P8. Thus, it is evident that this C-terminal B-cell epitope is naturally immunogenic in some human populations.

The Kenyan population had the highest responder frequency for anti-NANP antibodies. This finding is consistent with the higher sporozoite inoculation rates in western Kenya and the greater exposure of the Kenyan population to malaria parasites. The only peptide that was uniquely recognized by the Kenyan population was peptide P4, which is composed of three copies each of NANP and NVDP repeats. When the pattern of antibody reactivity to this peptide in the three populations was compared with the antibody reactivity to recombinant protein P11, which contains 30 NANP and 2 NVDP repeats, the trend of antibody responses against peptides P4 and P11 was found to be similar: both showed relatively higher reactivities in the Kenyan samples than in the Brazilian and PNG samples. This result suggests that the sensitivity of detecting antibody to the major B-cell epitope, the NANP repeat region, is dependent on the numbers of NANP units used as an antigen in the FAST-ELISA.

The NANP repeat region has been described as an immunodominant epitope because it is the most immunogenic epitope within the CS protein (4, 5, 10, 14, 17, 18). While this description may be consistent with the Kenyan serologic data, it is inconsistent with the serologic data from Brazil and PNG. The latter two populations have similar responder frequencies for epitopes localized within NANP repeat (P11) and non-repeat (P2 and P7) regions, a result suggesting that the NANP repeat region is not the only immunodominant epitope within the CS protein. As previous investigations have revealed, the antibody response to the NANP repeat region increased as a function of age in all three populations studied (4, 5, 10, 14, 17, 18). On the other hand, however, although the antibody responses to two other non-repeat-region-based B-cell epitopes (represented by P2 and P3 and by P7 and P8) in the PNG and Brazilian populations appeared to increase in an age-dependent manner, they were statistically significant only for P7 and P8 and not for P2 and P3. This finding indicates that the immune responses to all the epitopes may not increase as a function of age. Indeed, in a recent study, an inverse correlation between age and proliferative responses to some of the T-cell epitopes in the MSA-1 antigen of *P. falciparum* was found for a West African population (21).

This study also provided an opportunity to examine humoral responses against known T-cell determinants of the CS protein. As shown by the reactivity of antibodies to peptide P2, only the region I B-cell epitope, which is complementary to the Th1R T-cell determinant, induces a natural host antibody response. This result suggests that both antibody and T-cell pressure could enforce the maintenance of polymorphisms in the Th1R region. Compared with this natural host antibody response to Th1R, antibody responses to the helper T-cell determinant (Th2R) and the CTL-containing determinant (Th3R) were not observed. The low reactivity to peptides P9 and P10 was not due to poor binding of the peptides to plastic beads, because peptides P9 and P10 were recognized by some of the plasma or serum samples. However, the antibody responses to the Th2R and Th3R determinants may have been restricted by the host genetic background, so some other population in which malaria is endemic may recognize these determinants as B-cell epitopes.

In conclusion, this study confirms the existence of two non-repeat-region-based B-cell epitopes of the CS protein of *P. falciparum*, against which natural humoral immune responses are developed. We also showed that as far as reactivity to individual determinants is concerned, individuals from the three tested regions in which malaria is endemic mounted various antibody responses to different immunogenic determinants of the CS protein. Moreover, the CS protein repeat-sequence-based epitope is not the only immunodominant determinant of this vaccine antigen; natural exposure to sporozoites induces as strong an antibody response to the non-repeat-sequence-based B-cell determinants as to the repeat-sequence-based B-cell determinant. Information on the expression of host immune responses to CS protein determinants will be useful in planning vaccine development

strategies and epidemiologic studies to monitor the expression of natural host immunity against parasite antigens.

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