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## IMMUNE RESPONSES AGAINST *PLASMODIUM FALCIPARUM* ASEXUAL BLOOD-STAGE ANTIGENS AND DISEASE SUSCEPTIBILITY IN GABONESE AND CAMEROONIAN CHILDREN

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**Abstract.** The frequency and level of cellular and humoral responses to seven synthetic peptides from asexual blood stages of *Plasmodium falciparum* were measured in two cohorts of children living in areas highly endemic for malaria in Gabon and Cameroon. A prospective longitudinal study was conducted for one year in these sites to examine the relationship between specific *in vitro* immune responses and susceptibility to clinical malaria. Clinical protection was related to high proliferative responses (merozoite surface antigen-1 [MSA-1] and MSA-2 peptides) as well as to elevated antibody levels (schizont extract, MSA-2, and rhoptry-associated protein-1 [RAP-1] peptides) in the village of Dienga, Gabon. Higher response rates of interferon- $\gamma$  but lower response rates of tumor necrosis factor- $\alpha$  to four and six peptides, respectively, were observed in Dienga than in Pouma that were independent of the older age of the Gabonese children. Age accounted only for the higher prevalence rate in Dienga of the antibody responders to the peptide from Pf155/ring-infected erythrocyte surface antigen (RESA). Our results support the inclusion of epitopes from MSA-1, MSA-2, RAP-1, and Pf155/RESA antigens in a subunit vaccine against malaria, but show that a longitudinal clinical, parasitologic, and immunologic study conducted according to identical criteria in two separate areas may lead to contrasting observations, demonstrating the geographic limitation of the interpretation of such results.

Since many of the malaria vaccines currently under development are subunit vaccines reproducing isolated fragments of parasite antigens, it is important to characterize the function of the responses triggered by the T and B cell epitopes of candidate antigens. The histidine- and alanine-rich protein-2 (HRP-2) is released from infected erythrocytes. Although its immunologic importance in humans remains uncertain, impressive protection of monkeys was achieved using recombinant HRP-2 proteins.<sup>1,2</sup> Various merozoite surface antigens of *Plasmodium falciparum* are being prepared for inclusion in a subunit vaccine against the asexual blood stages of the malaria parasite, and among them, the merozoite surface antigens-1 and -2 (MSA-1 and MSA-2), the rhoptry-associated protein-1 (RAP-1), and the ring-infected erythrocyte surface antigen (Pf155/RESA) have been shown to be strong immunogens eliciting partial or total protection when tested in monkeys.<sup>3</sup> Several epidemiologic studies conducted in humans living in malaria-endemic areas suggested a protective role of the naturally acquired immunologic responses to MSA-1,<sup>4-7</sup> MSA-2,<sup>8</sup> and Pf155/RESA,<sup>9</sup> as well as an association between levels of IgG to RAP-1 and protection against high *P. falciparum* densities in blood.<sup>10</sup>

The individual susceptibility to malaria is influenced by numerous factors that include host genotype, parasite virulence, and specific immunity. In areas where malaria is endemic, the acquisition of anti-malarial immune protection is progressive, as assessed by a marked and gradual decrease after 5 years of age in mortality and morbidity attributed to this infection.<sup>11</sup> To investigate the influence of the acquired immunity to malaria on disease susceptibility, well-defined cohort studies are needed, in which malariometric indices are recorded longitudinally. In the present study, two cohorts of children were enrolled in two endemic regions of central

Africa where the epidemiology of malaria is different, as previously described.<sup>12</sup> The cellular and humoral reactivity to the specific *P. falciparum* antigens described earlier was measured in these cohorts in order to 1) investigate in each site the associations between specific immune responses and susceptibility to disease and 2) compare the immunologic status in children from both sites. Cellular reactivity was investigated by measurement of lymphocyte proliferation and cytokine release, the cytokines of interest being tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-10 (IL-10). Humoral reactivity was assessed by the level of plasma antibodies directed to *P. falciparum* asexual blood stages.

### SUBJECTS AND METHODS

**Study areas.** A fully equipped field base was established in 1994 in the village of Dienga, located in southeastern Gabon, which allowed full-time surveillance of study cohorts. This village of about 1,200 inhabitants is situated in the rain forest area, and malaria is highly endemic with peaks of transmission occurring during the rainy season from February to May and from October to December.<sup>13</sup> The other survey was performed during the same period in the village of Pouma located in central Cameroon 150 km west of Yaounde and 100 km east of Douala. The equatorial climate allows perennial transmission of *P. falciparum* with seasonal increases from April to June and from September to December.

**Subjects.** A clinical, biologic, and parasitologic follow-up was carried out from February 1995 to March 1996 (except during the summer school holidays) among the 300 children attending school in Dienga. Briefly, axillary temperature was



TABLE 1  
List of synthetic peptides from *Plasmodium falciparum* asexual blood-stage antigens

Antigens*	Sequence†	Residues	Abbreviation	Reference
MSA-1	LNDITKEYEKLLNEI	521-535	P1	15
	LESYKKFLPEGTD	1173-1185	P2	15
MSA-2	SNTFINNA	27-34	P3	16
	NTSDSQKE	213-220	P4	16
RAP-1	LTPLEELYP	202-210	P5	17
HRP-2	AHH(AHHAAD) <sub>2</sub>	171-185	P6	18
Pf55/RESA	K(EENVEHDA) <sub>4</sub>	892-924	P7	19

\* MSA-1 = merozoite surface antigen-1; RAP-1 = rhoptry-associated protein-1; HRP-2 = histidine and alanine rich protein-2; RESA = ring-infected erythrocyte surface antigen.  
† The sequences are given in one-letter code.

measured daily, and when the temperature was  $>37.5^{\circ}\text{C}$ , Giemsa-stained thick blood smears obtained by fingerprick were examined. An appropriate treatment was initiated when *P. falciparum* parasitemia exceeded  $400/\mu\text{l}$ . Every two weeks, thick blood smears were examined to detect asymptomatic parasitemia. Every month, evaluation of antimalarial self-medication was done by the detection of 4-aminoquinolines metabolites in urine using the Saker-Solomons test.<sup>14</sup> The same follow-up procedures were applied to the survey performed during the same period in the village of Pouma. A total of 186 school children were followed-up there from April to December 1995, covering the whole malaria transmission season. Clinical and parasitologic data allowed us to distinguish between protected and unprotected children. Protected children were defined as those who never presented during the entire survey with a febrile episode associated with either a *P. falciparum* parasitemia  $> 400/\mu\text{l}$  or a positive Saker-Solomons test result. Unprotected children were defined as those who presented at least one malaria attack defined by the association of fever and a *P. falciparum* parasitemia  $\geq 5,000/\mu\text{l}$ . Other children, those who presented with either a fever and a *P. falciparum* parasitemia between  $400/\mu\text{l}$  and  $5,000/\mu\text{l}$  or those with a fever, a *P. falciparum* parasitemia  $< 400/\mu\text{l}$ , and a positive Saker-Solomons test result, remained unclassified. Based on this, 76 children (mean age = 10.6 years, range = 6-17) from the cohort of Dienga, including 38 protected and 38 unprotected children were classified and selected for the immunologic study. Similarly, 43 school children (mean age = 8.8 years, range = 5-15) from the cohort of Pouma, including 32 protected and 11 unprotected children, were selected for the immunologic study. Blood was drawn at the end of the follow-up after informed consent was obtained from the parents of the children. Ethical clearance for the study was provided by the Ethics Committees in Gabon and Cameroon.

**Parasitologic measurements.** During the follow-up, 16 and 10 twice-a-month thick blood smears were prepared in Dienga and Pouma, respectively, for the detection of asymptomatic infections. After staining the smears with Giemsa, malaria parasites were counted against 1,000 leukocytes, and the mean geometric parasite density (MGPD) of positive slides was determined.

**Antigens.** Seven synthetic peptides were used that represented B and/or T cell epitopes from conserved and semi-conserved regions of *P. falciparum* asexual blood stages. These peptides, whose sequences are shown in Table 1, were in carboxy forms and were obtained from the Pasteur Institute (Paris, France). They were used at a final concentration

of  $1 \mu\text{M}$  in the *in vitro* cultures. Control antigens were the mitogen leukoagglutinin (final concentration =  $10 \mu\text{g/ml}$ ; Sigma, St. Louis, MO) and the recall antigen tuberculin-purified protein derivative (PPD, final concentration =  $10 \mu\text{g/ml}$ ; Statens Seruminstitut, Copenhagen, Denmark).

**Lymphocyte proliferative assay (LPA).** Within 16 hr after bleeding, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque (Pharmacia, Uppsala, Sweden), and cell viability was confirmed by trypan blue staining. Purified PBMC were suspended at a concentration of  $10^6$  cells/ml in buffered RPMI 1640 medium supplemented with gentamicin ( $25 \mu\text{g/ml}$ ), 2 mM glutamine, and 10% pooled human AB serum, and  $100\text{-}\mu\text{l}$  aliquots were plated in triplicate in flat-bottomed, 96-well plates. Control antigens, peptides, or culture medium alone were added in  $100\text{-}\mu\text{l}$  amounts at the indicated concentrations. Plates were incubated at  $37^{\circ}\text{C}$  in a humidified chamber containing 5%  $\text{CO}_2$ . After 6 days,  $110\text{-}\mu\text{l}$  culture supernatants were removed and  $50 \mu\text{l}$  of fresh medium containing  $0.5 \mu\text{Ci}$  of methyl- $^3\text{H}$ -thymidine (specific activity = 2 Ci/mmol; Amersham, Les Ulis, France) were added to each well. After an additional 16 hr, cells were collected on glassfiber filter paper and radioactivity was counted. Stimulation indices (SIs) were calculated by dividing the geometric mean counts/min of antigen-stimulated cultures by the geometric mean counts/min of unstimulated cultures. The threshold of positivity for all antigens was set at an SI  $> 2.0$  and geometric mean counts/min  $>$  the geometric mean + 2 SD of an individual's own background.<sup>20</sup>

**Cytokine assays.** Six-day culture supernatants from each triplicate sample were pooled and stored at  $-80^{\circ}\text{C}$ . Interferon- $\gamma$  was assayed in the undiluted supernatants using a two-site ELISA according to the manufacturer's instructions (Mabtech, Stockholm, Sweden). Similar culture plates were incubated for 72 hr, and triplicate supernatants were pooled and stored at  $-80^{\circ}\text{C}$  for assaying TNF- $\alpha$  and IL-10 contents, using a two-site ELISA (Pharmingen, San Diego, CA). Cytokine concentrations were determined by reference to standard curves prepared with recombinant human cytokines (Pharmingen). The thresholds of sensitivity were  $8 \text{ pg/ml}$  for TNF- $\alpha$  and  $2 \text{ pg/ml}$  for IFN- $\gamma$  and IL-10. For statistical purposes, values under the threshold were assigned a concentration of half this value. Mitogen- and antigen-induced cytokine production was derived from the difference between the cytokine content in stimulated cultures and the spontaneous cytokine content in unstimulated cultures.

**Antibody measurements.** Antibodies to *P. falciparum* in plasma were measured by ELISA using successively 1) a

schizont extract obtained by sonication of *in vitro* cultures of the Palo Alto strain of *P. falciparum* (7 µg/ml), 2) a 200-fold diluted plasma, and 3) an anti-human IgG (Fc specific) conjugated to alkaline phosphatase (Sigma). Bound enzyme was detected with *p*-nitrophenylphosphate and the absorbance was read at 405 nm. Antibodies directed against the P1, P3, P4, P5, and P7 peptides corresponding to the known B cell epitopes among the peptides under investigation, were tested by ELISA using the peptides conjugated to bovine serum albumin.<sup>21</sup> Reference positive and negative control plasmas were included in each plate, and results were expressed in arbitrary units (AU) calculated from the formula  $100 \times [\ln(A_{405} \text{ test plasma}) - \ln(A_{405} \text{ Pool -})] / [\ln(A_{405} \text{ Pool +}) - \ln(A_{405} \text{ Pool -})]$ .<sup>22</sup> The thresholds for positivity were set at 21.1 AU for anti-*P. falciparum* IgG, 19.1 AU for P1, 2.9 AU for P3, 7.7 AU for P4, 10.5 AU for P5, and 29.2 AU for P7, as determined from the mean reactivities + 2 SD of > 50 plasmas from nonimmune subjects.

**Statistical analysis.** Differences in proportions were analyzed using chi-square or Fisher's exact tests. Differences in means were tested by Student's unpaired *t*-test on linear or log-transformed values. When variable distribution was not normalized by log transformation, the nonparametric Mann-Whitney U test was used. Statview 4.5 (Abacus Concept, Berkeley, CA) was used for these calculations. The association between specific immune responses and demographic characteristics (age, living area) that were found to be significant in the univariate analysis was investigated by logistic regression analysis using BMDP software (University of California, Los Angeles, CA). For all tests, *P* values < 0.05 were considered significant.

## RESULTS

**Results obtained in Dienga.** Clinical and hematologic results are shown in Table 2. Sex, blood group, presence of the sickle cell trait, and of *P. falciparum* parasites at blood drawing were equally distributed among protected and unprotected children. As expected, protected children were older than unprotected ones and the MGPD was lower in protected children. Immunologic results retained for analysis concerned individuals whose PBMC proliferated in response to leukoagglutinin and/or PPD (99%). Cellular responsiveness to control antigens and to synthetic peptides are shown in Figure 1. The frequencies of the IFN-γ and IL-10 responses (> 20%, except for IFN-γ P7) were higher than those of LPA and TNF-γ responses (< 20%, except for LPA-P3, LPA-P4, and LPA-P5). Antibody response rates (Figure 2) were 97% for the schizont extract and > 18% for peptides. Statistically significant differences in the cellular and humoral responses in relation to protection are presented in Table 3. Protected children presented higher proliferative response rates after stimulation by P2 and P3 peptides than unprotected ones. The presence of detectable *P. falciparum* blood-stage parasites at the time of sampling was not related to protection but was associated with higher T cell proliferative responses in the presence of P2, P3, P5, and P6 (*P* from 0.006 to 0.02, by Student's unpaired *t*-test). Levels of antibody responses directed to the schizont antigen as well as to P4 and P5 were higher in protected children than in unprotected children.

TABLE 2  
Clinical and hematologic results in protected and unprotected children from Dienga, Gabon (n = 76) and Pouma, Cameroon (n = 43)\*

	Dienga		Pouma		P†
	Protected (n = 38)	Unprotected (n = 38)	Protected (n = 32)	Unprotected (n = 11)	
Mean ± SD age (years)	11.5 ± 2.6	9.6 ± 1.9	9.0 ± 2.6	8.3 ± 1.8	NS
Sex (ratio M/F)	0.81	1.53	0.52	0.37	NS
Blood group (A/B/AB/O)§	9/5/0/24	8/7/0/23	9/7/4/12	3/3/0/5	NS
Sickle cell traits	0.19	0.25	0.28	0.18	NS
<i>P. falciparum</i> -positive blood smears§	0.39	0.29	0.37	0.18	NS
MGPD (µM)¶	148 (108–202)	253 (168–382)	545 (414–717)	1,630 (890–2,983)	0.0024
					0.0003
					0.035
					0.013
					NS
					NS
					<0.0001

\* NS = not significant.

† *P* value of the chi-square test (sex, blood group, sickle cell trait, *P. falciparum*-positive blood smears), the Student's unpaired *t*-test (age), or the Mann-Whitney *U*-test (MGPD) between protected and unprotected children.

‡ *P* values determined as in † but between study sites.

§ Observed frequency or effective, *P. falciparum*-positive blood smears were determined on the blood drawn for the immunologic assays.

¶ MGPD is the mean geometric parasite density (95% confidence interval) of positive slides, calculated on the basis of 16 (Dienga) and 10 (Pouma) cross-sectional surveys.

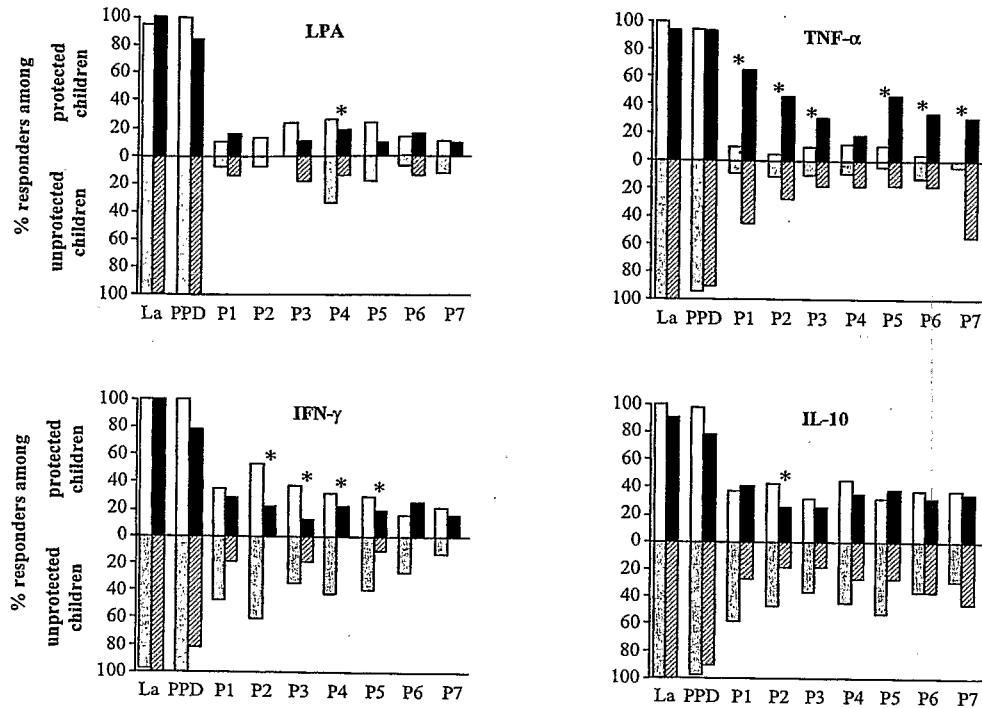


FIGURE 1. T cell response rates to leukoagglutinin (La), tuberculin-purified protein derivative (PPD), and synthetic peptides from *Plasmodium falciparum* blood-stage antigens in 76 children from Dienga, Gabon and 43 children from Pouma, Cameroon. Children from Dienga included 38 protected (open bars) and 38 unprotected (shaded bars) children, and children from Pouma included 32 protected (solid bars) and 11 unprotected (striped bars) children. Peptides are designated as in Table 1. Lymphocyte proliferation was measured by thymidine incorporation. Cytokine release was measured by an ELISA as the difference between stimulated and unstimulated culture supernatants. For thresholds of positivity, see Subjects and Methods. \**P* < 0.05, by chi-square test between children from both sites. LPA = lymphocyte proliferative assay; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; IFN- $\gamma$  = interferon- $\gamma$ ; IL-10 = interleukin-10.

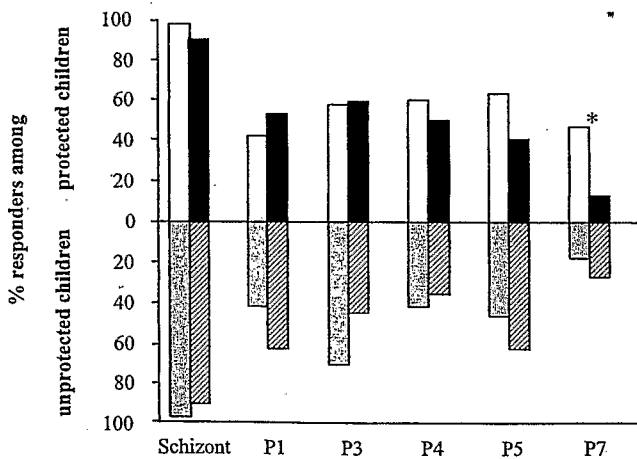


FIGURE 2. Antibody response rates to schizont extract and to synthetic peptides from *Plasmodium falciparum* blood-stage antigens in 76 children from Dienga, Gabon and 43 children from Pouma, Cameroon. Children from Dienga included 38 protected (open bars) and 38 unprotected (shaded bars) children, and children from Pouma included 32 protected (solid bars) and 11 unprotected (striped bars) children. Peptides are designated as in Table 1. Antibodies were measured by an ELISA. For thresholds of positivity, see Subjects and Methods. \**P* < 0.05, by chi-square test between children from sites.

TABLE 3  
Differences in immune responses to *Plasmodium falciparum* blood-stage antigens between protected (*n* = 38) and unprotected (*n* = 38) children from Dienga, Gabon\*

		% $\pm$	Geometric mean values of responders (95% CI)	<i>P</i> †
<b>Cellular responses</b>				
P2	LPA			
	Protected	10	2.4 (2.2–2.5)	0.045
Unprotected	5	2.6 (1.9–3.5)		
P3	LPA			
	Protected	24	2.6 (2.3–2.9)	0.005
Unprotected	0	0		
<b>Antibody responses</b>				
<b>Schizont</b>				
Protected		97	78.7 (73.3–84.5)	0.021
Unprotected		97	66.3 (59.7–73.5)	
P4	Protected	61	50.4 (39.4–64.4)	0.028
	Unprotected	42	33.2 (22.2–49.5)	
P5	Protected	63	42.0 (33.5–52.6)	0.026
	Unprotected	47	27.3 (20.7–35.8)	

\* Peptides are designated as in Table 1. Lymphocyte proliferation (LPA) was measured by thymidine incorporation and expressed as the stimulation index. The presence of IgG antibodies was determined by ELISA and expressed as arbitrary units. CI = confidence interval.

† Percentages of responders among protected or unprotected groups of children.

‡ *P* values were determined from all subjects from protected and unprotected groups, using the Student's unpaired *t*-test (LPA) and the Mann-Whitney *U* test (antibody).

**Results obtained in Pouma.** The distribution of clinical and hematologic parameters was identical in the protected and unprotected groups, except for the MGPD, which was lower in protected children (Table 2). Figure 1 shows that the frequencies of the TNF- $\alpha$  and IL-10 responses ( $> 20\%$ , except for TNF- $\alpha$  P4) were higher than those of LPA and IFN- $\gamma$  responses ( $< 20\%$ , except for IFN- $\gamma$  P1). Antibody response rates (Figure 2) were 91% for the schizont extract and  $> 12\%$  for peptides. No difference in the cellular nor in the humoral responsiveness appeared between protected and unprotected groups, but the weakness of the statistical analysis, given the small number of individuals in Pouma, has to be taken into account.

**Significant differences in immune responses between the sites.** Children were older in Dienga than in Pouma (Table 2), and this difference was observed both between protected and unprotected groups ( $P = 0.0002$  and  $P = 0.04$ , respectively, by Student's unpaired *t*-test). This observation may partially explain the differences revealed in the parasitologic and immune status of both cohorts. The children from Dienga presented a lower MGPD than those from Pouma (Table 2), and again this difference was reflected both between protected and unprotected groups (both  $P < 0.0001$ , by Mann-Whitney U-test). Higher frequencies of IFN- $\gamma$  producers to P2, P3, P4 and P5 were recorded in Dienga (Chi-square test, all  $P < 0.03$ ), while higher frequencies of TNF- $\gamma$  producers to all peptides except P4 were observed in Pouma (all  $P < 0.001$ , by chi-square test), as highlighted in Figure 1. This observation was not related to the older age of children from Dienga compared with Pouma (all  $P \geq 0.05$ , by logistic regression analysis). Anti-P7 antibody responders (Figure 2) were more frequently encountered in Dienga than in Pouma ( $P = 0.04$ , by chi-square test). The difference of age between Gabonese and Cameroonian children influenced this observation because the rate of anti-P7 antibody responders increased with age (logistic regression analysis, odds ratio = 1.26, 95% confidence interval = 1.06–1.54;  $P = 0.012$ ). Higher levels of antibodies to schizont extract and to P7 were found in Dienga compared with Pouma ( $P = 0.002$  and  $P = 0.0001$ , respectively, by Mann-Whitney U test).

#### DISCUSSION

The *P. falciparum* asexual blood stage antigens MSA-1, MSA-2, RAP-1, HRP-2 and Pf155/RESA contain T cell and B cell conserved epitopes. In the present study, we investigated cellular and humoral responses to a variety of synthetic peptides representing major T cell and/or B cell epitopes from these antigens in 76 and 43 school children living in areas highly endemic for malaria in Gabon and Cameroon, respectively. All synthetic peptides were able to induce a cellular response in some individuals. Overall, a proliferative response was observed in 5–30% of the donors in the presence of any given peptide (except for P2 in Pouma). Release of IFN- $\gamma$  was observed in 7–56% of peptide-stimulated cultures. Higher proliferative response rates but similar frequencies of IFN- $\gamma$  responders have been reported for P1 in Indian adults and for P7 in Thai adults.<sup>23, 24</sup> Overall, each peptide induced a cellular response, as assessed by proliferation or production of either cytokine, in a mean of 66%

and 59% of children from Dienga and Pouma, respectively. A cellular response to at least one tested peptide was obtained in 95% of donors from both sites, indicating the presence of effector specific anti-malarial immune responses for 113 of the children.

The frequency of *P. falciparum* infections has been found to be twice as high in Pouma as in Dienga, whereas the malaria attack rate was twice as high in Dienga compared with Pouma.<sup>12</sup> The differences in T cell reactivity, as reflected by inverse profiles of IFN- $\gamma$  and TNF- $\alpha$  secretions in Dienga and Pouma, may be related to this clinical observation. In contrast to its toxicity towards exo-erythrocytic stages of malaria parasites, IFN- $\gamma$  has no direct effect on intra-erythrocytic parasites, but may contribute to immunity by enhancing phagocytosis of parasitized and IgG-opsonized erythrocytes.<sup>25</sup> However, production of IFN- $\gamma$  may also contribute to increase the production of TNF- $\alpha$  associated with pathology. A strong negative correlation between age and plasma TNF- $\alpha$  levels has been previously documented in a Gabonese cohort, and the explanations proposed by the investigators were an age-related antibody response to TNF- $\alpha$ -inducing malaria antigens, as well as an age-related decrease in parasite load.<sup>26</sup> In the cohort from Dienga, protection was positively associated with the strength of proliferative responses to peptides P2 (a conserved epitope from the N terminus of MSA-1) and P3 (a semi-conserved epitope from the C terminus of MSA-2). Since age and proliferative response rates to these peptides were not related, this association cannot be attributed to the fact that protected children were older. Lastly, two previous studies that investigated the protective value of cellular response to RESA synthetic peptides did not report any association between the presence or level of cellular responses (T cell proliferation and IFN- $\gamma$  release) to RESA peptides and subsequent malaria morbidity.<sup>27, 28</sup> The current study confirms these data and extends them to additional markers of cellular activation.

Environmental factors may account for the observed differences between the sites. A difference in intensity or seasonality of *P. falciparum* transmission may induce different reactivities to malarial antigens in the two sites. The genetic diversity of the *P. falciparum* circulating strains may also influence the immune disparities between the sites.<sup>29</sup> Indeed, the polymorphisms of the MSA-1 and MSA-2 genes from *P. falciparum* have been found as high in Dienga as in Pouma, although several alleles were observed in one site<sup>30</sup> but not in the other (Ntoumi F, unpublished data). An age-dependent acquisition of the immune responses to these antigens may reflect cumulative exposure to different polymorphic forms of MSA-1 or MSA-2. Differences in *P. falciparum* strains circulating in both sites may also involve several phenotypic characters that induce variable levels of TNF production, as has previously been observed for laboratory parasite lines.<sup>31</sup>

As usually observed in areas highly endemic for malaria, more than 90% of the children presented with antibodies to schizont extract, with higher antibody levels in Dienga than in Pouma.<sup>8</sup> In Dienga, the level of antibodies to this antigen was related to clinical protection and responders were older than non-responders. The antibody response rates to peptides P1 to P7 are in agreement with previous studies conducted in children having not totally acquired their natural immu-

nity.<sup>8,10,28</sup> Similar to the previous finding of an association between levels of IgG reactivities to recombinant RAP-1 and protection against high *P. falciparum* densities,<sup>10</sup> we observed that the levels of antibodies to peptides P5 (RAP-1) and P4 (MSA-2) were associated with clinical protection in Dienga. The influence of age on the presence of antibody reactivity to the recombinant MSA-2 and Pf155/RESA proteins has already been demonstrated, and the levels of specific antibodies to MSA-2 and Pf155/RESA have also been related to reduced malaria morbidity.<sup>8,28,32</sup> Our results confirm this relationship between age and the antibody response rate to P7.

The results presented here illustrate the difficulty of interpreting immunologic results according to the site where they are recorded. Indeed, clinical protection was related to high proliferative responses (MSA-1 and MSA-2 peptides) and antibody levels (schizont extract, MSA-2, and RAP-1 peptides) in the village of Dienga, Gabon. No such relationship was observed in Pouma. Higher frequencies of IFN- $\gamma$  responses but lower frequencies of TNF- $\alpha$  responses to almost all peptides were observed in Dienga that were independent of the older age of the Gabonese children. The complexity of interpretation of these results reinforces the necessity to conduct longitudinal studies to measure the relevant indices of clinical protection to be related to the anti-malarial immune responses. Nevertheless, the cellular and humoral specific responses described in this paper support the inclusion of epitopes from MSA-1, MSA-2, RAP-1, and Pf155/RESA into a subunit vaccine against malaria.

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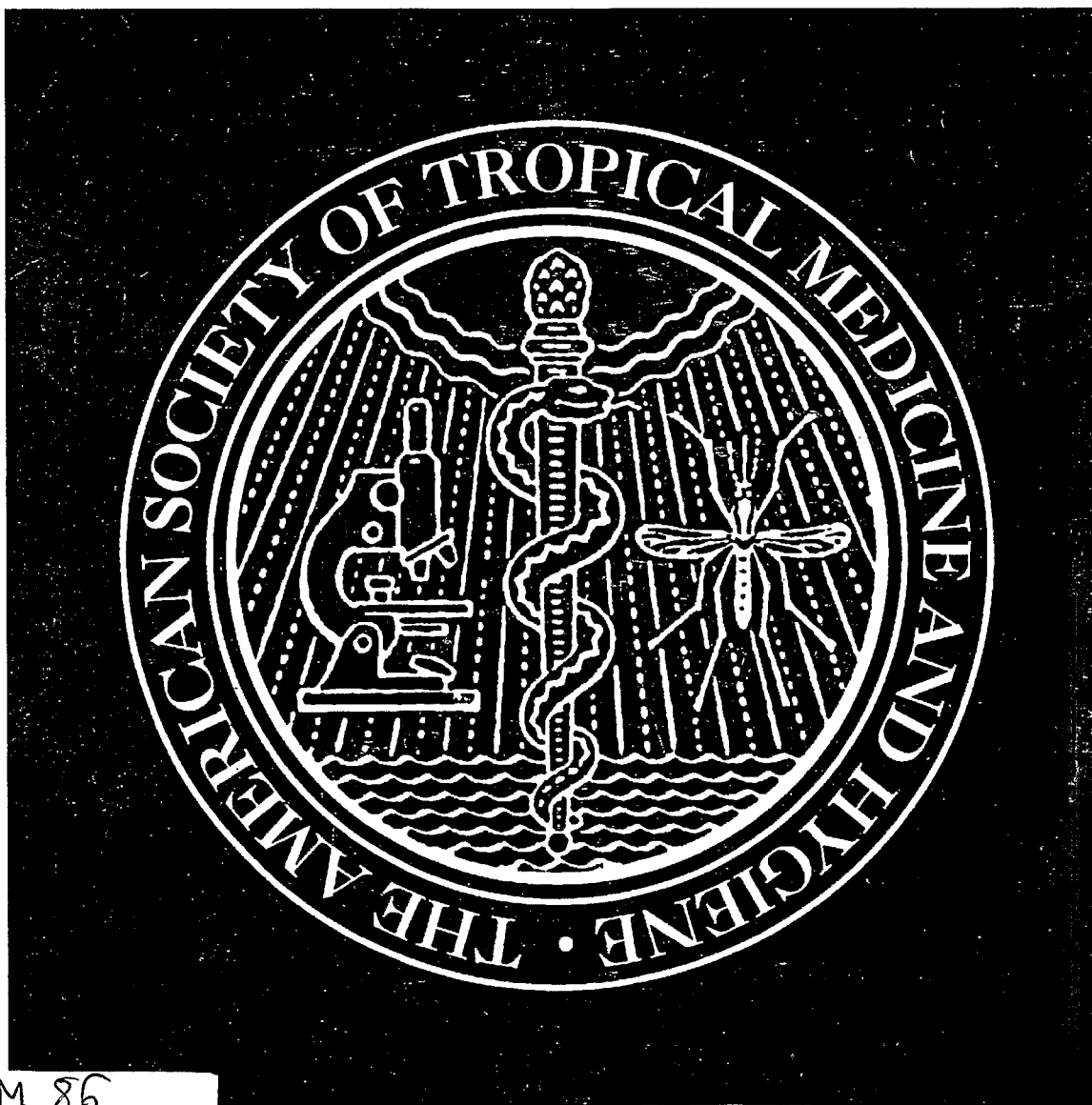


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