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Identification of T-Cell Determinants in Natural Immune Responses to the *Plasmodium falciparum* Apical Membrane Antigen (AMA-1) in an Adult Population Exposed to Malaria

ALTAF A. LAL,[1]* MOLLY A. HUGHES,[1,2] DENISE A. OLIVEIRA,[1] CECILIA NELSON,[1] PETER B. BLOLAND,[1] AGGREY J. OLOO,[3] WILLIAM E. HAWLEY,[1,3] ALLEN W. HIGHTOWER,[1] BERNARD L. NAHLEN,[1,3] AND VENKATACHALAM UDHAYAKUMAR[1]

Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia[1]; Vanderbilt University School of Medicine, Nashville, Tennessee[2]; and Vector Biology and Control Research Center, Kenya Medical Research Institute, Kissian, Kenya[3]

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AMA-1 of *Plasmodium falciparum* is a promising candidate antigen in malaria vaccine development. In this study, we have mapped the immunodominant T-cell determinants in this antigen by using synthetic peptides. From the amphipathic scores, 17 putative T-cell determinants were identified. Nine of the 17 peptides complementary to the putative T-cell determinants induced proliferation of peripheral blood mononuclear cells (PBMC) from Kenyan residents who had lifelong exposure to malaria; none of these peptides induced proliferation of PBMC from donors who were not previously exposed to malaria. This indicates that AMA-1 peptides were stimulating T cells that were previously primed by prior exposure to *P. falciparum*. Many positive responders showed reactivity to more than one peptide, and some of the potent proliferative T epitopes were found to be localized in the highly conserved regions of AMA-1, suggesting that it may be possible to induce T-cell memory that can recognize different variant forms of the parasite. This information on the natural immune responses against the AMA-1 vaccine antigen in clinically immune adults will be helpful in the development of an AMA-1 antigen-based malaria vaccine and may also guide testing of AMA-1-based vaccine formulations.

Malaria remains a major public health problem, affecting 300 to 500 million people globally, with an estimated annual mortality rate of about 2 million among children in Africa alone (13). The traditional malaria control approaches have not been successful because of the emergence of drug-resistant parasites and insecticide-resistant mosquito vectors. Therefore, efforts are under way in several laboratories to develop a vaccine directed primarily against *Plasmodium falciparum*, which causes most of the mortality associated with malaria.

Several antigens specific to different stages of *P. falciparum* have been cloned and immunologically characterized (reviewed in reference 21). Among the blood-stage antigens identified, apical membrane antigen 1 (AMA-1) has emerged as one of the leading target vaccine candidates (1a). The AMA-1 of *P. falciparum* is an 83-kDa polypeptide with characteristics of an integral membrane protein (22). AMA-1 contains a hydrophobic signal sequence of 23 amino acids in the C-terminal region that correspond to a transmembrane domain and has a 55-amino-acid cytoplasmic tail (1a). AMA-1 is synthesized during late schizogony and localizes in the electron-dense neck of the rhoptries (6). This protein is processed to a 66-kDa protein which may be carried on the surface of mature merozoites (6). The molecule may be involved in merozoite release and invasion of erythrocytes (6, 22, 28, 32).

The gene coding for the AMA-1 molecule is 1.8 kb in length and has been cloned and sequenced from human, monkey, and rodent malaria parasites (4, 18, 22, 23, 32). Unlike most sequenced *P. falciparum* antigen genes, the AMA-1 gene lacks repetitive sequences. Comparison of the sequences of AMA-1 genes from different species of malaria parasites shows that certain structural domains are highly conserved in all AMA-1 genes characterized (1a). Sixteen cysteine residues in the ectodomain of all the AMA-1 sequences are highly conserved (4, 32). The AMA-1 of *P. falciparum* differs from that of all other species in that it has an additional 54 amino acid residues near the N-terminal region of the protein (1a, 22). Genetic variation of this protein among different strains of *P. falciparum* has been examined. The results of these studies reveal that the variation is restricted to certain domains (21a, 22, 29), implying that the variable domains in the antigen may harbor immunologically sensitive epitopes.

Earlier immunological studies conducted with *Plasmodium knowlesi* AMA-1 demonstrated that invasion-inhibiting antibodies can be induced by the native AMA-1 of this parasite (7, 28). Later vaccination trials in rhesus monkeys demonstrated effective immunity against a *P. knowlesi* challenge (8). Antibodies from the protected monkeys showed in vitro inhibition of merozoite invasion into rhesus erythrocytes. It was also observed that protective antibodies recognized conformation-dependent epitopes. The fact that Fab fragments were more effective than the intact immunoglobulin G in blocking reinvasion of erythrocytes by merozoites in vitro strongly suggests a functional role for the AMA-1 protein in invasion (28).

The results from recent vaccine trials in a rhesus monkey model with *Plasmodium fragile* (5) and in a rodent model with *Plasmodium chabaudi* (1) lend further support for the potential of AMA-1 as a vaccine candidate. Immunization of mice with recombinant proteins of *P. chabaudi* containing only the ectodomain of AMA-1 gave complete protection against a subsequent challenge with *P. chabaudi*. Transfer of sera collected from these mice also provided protection against a similar challenge. Similarly, four of the five monkeys vaccinated

* Corresponding author. Mailing address: Mail Stop F-12, Centers for Disease Control and Prevention, 4770 Buford Hwy., Chamblee, GA 30341-3717. Phone: (404) 488-4047. Fax: (404) 488-4454.

with AMA-1 antigen from *P. fragile*, which was expressed in a baculovirus system, developed parasitemia upon challenge with *P. fragile* but recovered without treatment. One of the immunized monkeys and four of the control monkeys had high parasitemia and required treatment. In this trial, there was also a correlation between the antibody responses to AMA-1 and protection (5).

The ultimate test of a malaria vaccine will be determined in field trials under natural conditions of parasite exposure. Our continuing, longitudinal studies of malaria are aimed at delineating the characteristics of natural immunity to malaria infection and clinical illness. In the present study, our focus has been to characterize naturally immunogenic T-cell determinants of the AMA-1 vaccine antigen. We demonstrate here that the AMA-1 antigen contains at least six major T-proliferative epitopes.

The residents from 15 villages located near Lake Victoria (Asembo Bay) in western Kenya participated in this study. Malaria is transmitted year-round in this area. Transmission is highest during and following the long rains (March to May), with a second peak of transmission occurring after the short rains from October to December. Entomologic inoculation rates as high as 300 infected bites per year have been measured in this area (3, 13a). A total of 147 volunteers were tested, 73 women and 74 men. Of these volunteers, 137 were between the ages of 16 and 65 years, 1 was 15 years old, and 9 were adults who could not accurately recall their ages. Among these donors, 122 were uninfected at the time of the study, 24 were positive for *P. falciparum* infection, and the parasitemia status for 1 was unknown. None of the parasitemic donors were clinically ill. The parasite densities in these individuals were low, with a geometric mean parasite density of 0.00449% (range, 0.0004 to 0.1208%). Venous blood samples (10 to 15 ml) were obtained from the volunteers, and informed consent was obtained from all study participants. The study began in January 1994 and continued until July 1994, thus overlapping a prerainy and a rainy season. The peripheral blood mononuclear cells (PBMC) obtained from adult U.S. residents who had never traveled to known malarious regions were used as a negative control.

Peptides were synthesized by f-moc chemistry (2) at the Biotechnology Core Facility, National Center for Infectious Diseases, Centers for Disease Control and Prevention. Peptides were 80 to 90% pure and used without further purification. RPMI 1640 medium (GIBCO-BRL, Grand Island, N.Y.) supplemented with 5% fetal bovine serum and 5% normal AB^+ human serum (from donors not previously exposed to malaria), 2 mM glutamine, and 100 U of penicillin and streptomycin per ml was used.

Blood samples were collected aseptically in heparinized tubes in the field and transported under cold conditions to the laboratory within 2 h. PBMC were isolated on 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 serological studies. The PBMC at the interphase were collected, washed three times in 0.85% normal saline, and resuspended in culture medium. Viable PBMC counts were made under a phase-contrast microscope by the trypan blue dye exclusion test. A total of 200,000 PBMC in 100 μl of culture medium were added to each well of a flat-bottomed 96-well plate (Costar, Cambridge, Mass.). Various concentrations of peptides (3, 10, and 30 μg/ml) were added in 100 μl of culture medium.

Purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark), and phytohemagglutinin (PHA; 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 then labeled with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci [74.0 GBq] per mmol) (DuPont, NEN Research Products, Boston, Mass.) overnight. Cultures were harvested with a Skatron harvester, and the radioactivity was determined in a liquid scintillation counter. The geometric mean counts per minute (cpm) for each set of quadruplicate wells was calculated. The stimulation index (SI) was determined as the geometric mean cpm of peptide-stimulated culture divided by the geometric mean cpm of the unstimulated culture (background) as described before (30).

TABLE 1. Sequences and locations of peptides in AMA-1 antigen of *P. falciparum*[a]

Peptide	Amino acid sequence	Position of amino acids in AMA-1 protein
PL166	IVERSNYMGNPWTEYMAR	99–116
PL167	MRHFYKDNKYVKNLDELT	198–215
PL168	SKNVVDNWEKVCPRKNL	267–284
PL169	DGNCEDIPHVNEFSAIDL	317–334
PL170	EDIPHVNEFSAIDLFECN	321–338
PL171	DQPKQYEQHLTDYEKIKEG	348–366
PL172	SLYKNEIMKEIERESKRI	444–461
PL173	GNAEKYDKMDEPQHYGKS	571–588
PL186	EFTYMINFGRGQNYWEHPYQKS	14–35
PL187	INEHRPKEY	41–51
PL188	NLFSSIEIVERS	92–103
PL189	PLMSPMTLDEMRHFYKD	188–204
PL190	SRHAGNMIPDND	218–229
PL191	NGPRYCNKDE	259–271
PL192	AKDISFQNYT	279–288
PL193	YKSHGKGYNWGNY	390–402
PL194	EYKDEYADIPEH	527–538

[a] All the peptides represent amino acid sequences deduced from the genetic sequence of the FC27 isolate.

The putative T-cell epitopes within the AMA-1 molecule were predicted by using a computer algorithm (17). We found that there were eight sequences with an amphiphilic score of >15 and nine amino acid sequences with an amphiphilic score of between 5 and 15. Synthetic peptides corresponding to these sequences are listed in Table 1. Among these peptides, PL166, PL171, and PL194 represent highly conserved regions of AMA-1, while the remaining peptides represent sequences with one or more polymorphic amino acid residues. The sequence of PL194 represents part of the transmembrane domain and PL173 represents a cytoplasmic domain of AMA-1 antigen. All other peptides represent sequences from extracellular domains of AMA-1.

The ability of synthetic peptides complementary to the putative T-cell epitopes to induce proliferation of PBMC from persons naturally exposed to malaria was determined. As a positive control, PHA and PPD were also used to stimulate the PBMC. All the donors who were tested in this study showed a positive response to PHA, and about 90% of them also responded to PPD.

Subjects who showed an SI of >5 for each of the peptides tested were considered positive responders. Each donor was tested with at least three concentrations of the test peptides. Most of the positive responders showed proliferative response to more than one concentration of peptide tested. While most of the donors showed maximal SI with lower peptide concentrations (3 or 10 μg/ml), some donors needed a higher peptide

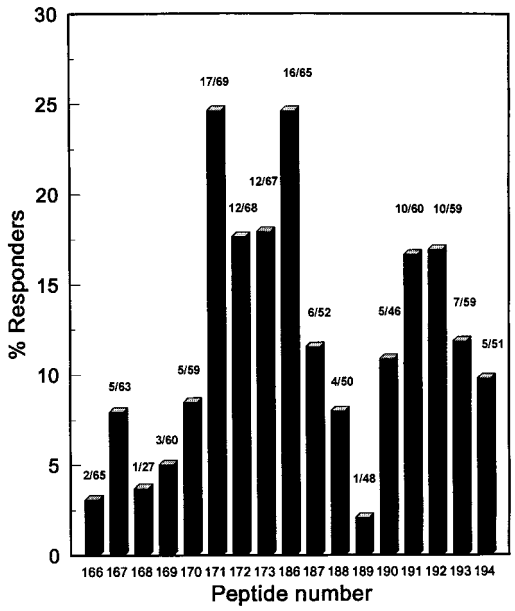

FIG. 1. Proliferative responses to synthetic peptides representing putative T-cell epitopes in the AMA-1 antigen of *P. falciparum*. PBMC from Kenyan men and women were isolated and stimulated with three different concentrations (3, 10, and 30 μg/ml) of synthetic peptides. Donors who showed an SI of >5 were scored as positive responders. The total number of responders/total tested for each peptide is given above each bar.

concentration (30 μg/ml) to achieve the highest stimulation. Therefore, subjects with an SI of >5 for at least one of the three concentrations of peptides were scored as positive responders. The results from these studies are summarized in Fig. 1. The degree of response to these peptides varied (Fig. 1). Peptides PL171 and PL186 were the highest stimulators, inducing proliferative response in about 25% of donors. PL172, PL173, PL191, and PL192 induced a positive proliferative response in about 16 to 18% of donors. About 10 to 12% of donors responded to PL187, PL190, PL193, and PL194. Only about 3 to 8% of the donors responded positively for the remaining peptides tested (PL166, PL167, PL168, PL169, PL170, PL188, and PL189), which may indicate that these peptides are weak T epitopes or that the immune responses to these determinants are genetically restricted. It is evident from this study that peptides PL171, PL186, PL172, PL173, PL191, and PL192 represent immunogenic T epitopes recognized by residents of western Kenya who are naturally exposed to *P. falciparum* infection.

The SI for each of the peptides in individual donors is shown in Fig. 2. The SI ranges from 0.1 to 86 with different peptides in these donors; however, in most of the donors, the SI was frequently <5. This SI (5 and above) was used as a cutoff value to score positive responders. In the positive responders, the SI often varied from 5 to 20 for most of the immunodominant peptides, and it rarely exceeded 20. This finding suggests that immunodominant epitopes in AMA-1 antigen are capable of eliciting strong memory responses.

We also found that the majority of positive responders showed positive reactivity to more than one peptide (Table 2). However, donors rarely responded to all the peptides that were simultaneously tested. Interestingly, those donors who responded to PL171, a major immunodominant epitope, were also found to respond (70% positive prevalence) to PL186, another major immunodominant epitope. Although many donors responded to several peptides, the magnitude of the response to each peptide was different. This heterogeneity in the pattern of responsiveness to these peptides is consistent with restriction of T-cell responses by HLA molecules.

TABLE 2. Proliferative responses to different immunodominant peptides in a subset of individual donors

Donor no.	SI						
	PL171	PL172	PL173	PL186	PL191	PL192	PL193
1579	5.52	31.34	14.98	7.87	1.31	0.95	3.29
1580	4.86	6.07	7.48	5.27	1.86	3.84	2.09
1581	38.88	19.53	16.38	14.09	6.39	5.0	6.02
1582	7.86	3.11	3.85	30.22	7.98	7.47	3.77
1583	5.81	7.04	3.39	22.94	2.40	2.11	1.83
1587	1.52	1.02	3.52	2.98	19.7	6.7	2.27
1590	1.82	2.19	2.98	4.45	5.49	8.17	4.47
1594	1.63	6.68	5.90	1.39	0.95	2.86	0.84
1595	13.36	86.47	3.38	2.86	7.03	1.53	3.42
1596	10.67	4.80	10.29	5.48	7.18	4.70	5.03
1597	5.12	4.04	7.92	6.86	4.38	3.76	2.4
1616	7.03	18.35	1.23	9.93	6.88	6.60	1.82

In order to determine if the parasitemic status of the donors has any relevance to T-cell proliferative responses to AMA-1

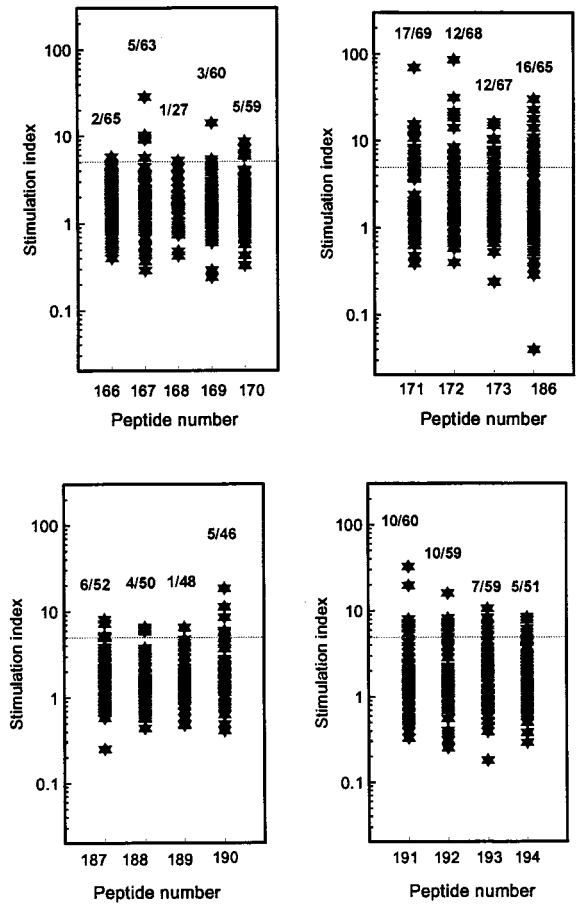

FIG. 2. Magnitude of proliferative responses in individual donors to peptides of AMA-1. The individual datum points were obtained from the same experiment shown in Fig. 1. The individual SI values for each individual tested are given. The numbers above each set of datum points represent the number of respondents with an SI of >5 per number of subjects tested.

TABLE 3. Proliferative responses to AMA-1 peptides in parasitemic and aparasitemic individuals[a]

Peptide	No. of responders/no. tested (% responders)	
	Aparasitemic individuals	Parasitemic individuals
171	16/58 (27.5)	1/11 (9.09)
172	11/57 (19.2)	1/11 (9.09)
173	11/56 (19.6)	1/11 (9.09)
186	16/56 (28.57)	0/9 (9.09)
187	5/42 (11.90)	0/10 (0)
188	2/40 (5)	2/10 (20)
189	1/39 (2.56)	0/9 (0)
190	4/36 (11.11)	1/9 (11.11)
191	10/53 (18.86)	0/7 (0)
192	9/52 (17.3)	1/7 (14.2)
193	7/52 (13.46)	1/7 (14.2)
194	4/46 (8.69)	0/5 (0)

[a] Responders are individuals who showed an SI of >5 to at least one of the tested concentrations of peptides. The data shown in Fig. 1 are divided into parasitemic and aparasitemic groups. Parasitemia was determined when the blood was drawn for the proliferation assay.

peptides, we compared T-cell proliferation to the peptides in aparasitemic and parasitemic persons (Table 3). The frequency of responses to the immunodominant peptides (PL171, -172, -173, -186, and -191) were generally higher in the aparasitemic group than in the parasitemic group (Table 3); however, there were no apparent differences in the SIs between the parasitemic and aparasitemic individuals (data not shown). Since the number of parasitemic subjects was small (16%), we could not determine whether this difference was statistically significant, and further studies are needed to confirm this observation. The frequency of responders to the rest of the peptides, most of which were poor stimulators of T-cell proliferation, was similar in the parasitemic and aparasitemic groups.

We also determined whether PBMC from U.S. residents who never traveled to malaria-endemic regions can respond to AMA-1 peptides in T-cell proliferative assays. None of these 15 volunteers showed an SI of >5 to any of the peptides (data not shown). The SI in this group of donors rarely exceeded 2, which indicates that AMA-1 peptides fail to stimulate naive T lymphocytes and that prior exposure to malaria parasites is necessary for mounting T-cell responses to AMA-1.

The potential of AMA-1 antigen of *P. falciparum* as a vaccine candidate has been strengthened by promising vaccine trials conducted in monkeys and rodents (1, 5, 8). In this study, we have demonstrated the presence of several potential T-helper cell epitopes in the AMA-1 molecule; these epitopes are widely recognized by subjects naturally exposed to malaria. We recognize that an optimal way to characterize all epitopes in any antigen is by using overlapping peptides that span the entire molecule, but this is expensive and laborious, and therefore, we chose to use a computer algorithm to predict the location of T-cell determinants (17). In our previous studies with malarial antigens, we have found that nearly 66% of the epitopes identified by computer algorithm tested positive in T-cell proliferation assays (30). These observations encouraged us to use the amphipathic score from the computer algorithm to predict putative T-cell epitopes in AMA-1. In addition to the use of a computer algorithm to predict T-cell determinants, several groups have exploited major histocompatibility complex (MHC) binding motifs to identify T-cell epitopes. Although this strategy has been used successfully in mapping MHC class I-restricted epitopes, it has not become widely useful in mapping MHC class II-restricted epitopes because of the lack of complete information on the binding motifs (12). Furthermore, the MHC binding motif approach may not be practical in defining epitopes at the population level because of extensive polymorphism in the HLA molecule.

We have shown that there are several T-cell epitopes (at least nine) in the AMA-1 antigen that can induce a proliferative response in persons who are naturally exposed to malaria. Furthermore, AMA-1 peptides induce a higher magnitude of T-cell proliferative responses in Kenyan residents but failed to induce positive proliferative responses in the uninfected human volunteers who had not been previously exposed to malaria. These observations suggest that the AMA-1 peptides stimulate T cells previously primed to AMA-1 in the Kenyan adults by lifelong exposure to *P. falciparum*. This is contrary to the findings in the case of the circumsporozoite protein, to which the T cells of persons who had no prior exposure to malaria parasites were shown to proliferate (27, 33), and some of these epitopes rarely induced strong T-cell responses in Papua New Guineans who were exposed to malaria (11). Although several factors, such as differences in the genetic background of the human populations and differences in the endemicity, could account for some of these results, other factors may also be important. For instance, unlike other repetitive-sequence-rich malarial antigens, AMA-1 does not contain repeat sequences. It has been argued by some investigators that multiple repeats in the malarial antigen, like CS protein, can promote T-cell-independent B-cell activation by direct cross-linking of antigen receptors on B cells (25), and it is known that T-cell-independent antigens are poor stimulators of T-cell memory (20). In this respect, it is unlikely that the AMA-1 molecule, which lacks antigenic repeats, can directly activate B cells in the absence of cognate T-cell–B-cell interaction and thereby promote the development of strong T-cell memory response. This hypothesis, however, needs further experimental validation.

Immunogenicity and antigenic diversity are two important considerations in malaria vaccine development. The AMA-1 molecule contains T-cell epitopes that are highly immunogenic, and some of these epitopes are located in highly conserved regions with limited amino acid polymorphism. We have determined that 9 of the 17 peptides which were recognized as putative T epitopes based on their amphipathic scores are capable of inducing T-cell proliferative responses at a higher frequency in persons who have been naturally exposed to malaria. Two of these peptides, PL171 and PL186, were found to be the most frequent stimulators of proliferative response, indicating that they are immunodominant T-cell epitopes in this protein. This finding is consistent with a previous report that showed that a peptide with an amino acid sequence complementary to that of PL171 was found to activate T-cell clones generated from malaria-exposed donors (24). It is important to note that the sequence represented by peptide PL171 is located in a nonpolymorphic region of the molecule, and another sequence, represented by peptide PL186, is located in a region with limited polymorphism. PL186 has 22 amino acids, and position 34 (K to N substitution) is variable in some of the laboratory and Kenyan field isolates (21a, 29). Recently, we have found that methionine at position 18, which was found to be conserved in all the laboratory isolates sequenced, is changed to threonine in one of the Kenyan isolates (21a). It remains to be determined whether these amino acid substitutions have any immunologic significance. PL194 is another peptide that represents a nonpolymorphic region but elicits substantial T-cell reactivity. PL166 is the only peptide among the three highly conserved peptides that induces poor T-cell proliferative response.

Among other predominant T-cell epitopes (PL172, PL173, PL191, and PL192), PL192 represents the most variable region, containing a three-amino-acid substitution. The variable amino acids in this peptide are located in proximity to each other, and one of the substitutions involves a charge change (Q to E change at 285). PL172 and PL173 have substitutions in two positions, and some of these changes involve charged amino acid residues. PL191 contains a variation in a single amino acid residue which is located at the C-terminal end. PL167 and PL189 are two peptides that contain multiple amino acid variations in their sequence, and both peptides are poor T-cell stimulators. Although the AMA-1 antigen has amino acid variations in several positions, it is unclear whether immune pressure alone is responsible for this selection. For example, variation is found not only in the ectodomain but also in the cytoplasmic domain of AMA-1. PL173, which was found to be a dominant T-cell and B-cell epitope, is localized in the cytoplasmic domain. At least two amino acid variations have been found within PL173, and it is unclear whether immunologic pressure has played a role in this selection. Irrespective of the mechanism responsible for the selection and maintenance of polymorphism in malarial antigens, it is important to determine the extent to which the polymorphism will affect vaccine efficacy. Unlike the CS protein, however, which contains several variable residues within the T-cell epitopes that are immunologically sensitive (9, 10, 16, 19, 26, 31), polymorphism in the AMA-1 antigen appears to be limited.

Studies of natural immune responses to malarial antigens, similar to the present study, will provide important baseline immunologic information on vaccine antigens. These immunoepidemiologic studies may also aid vaccine development efforts by identifying naturally immunogenic targets for immune intervention. Similar to studies of humoral immune responses in persons naturally exposed to malaria, field-based, cell-mediated studies can also provide important information. T-cell proliferation studies, as shown here, identify the immunodominant epitopes of a vaccine antigen. The T-cell epitopes from different malarial antigens can be then incorporated in a multiepitope vaccine to ensure maximal boosting of immune responses by parasite exposure. In addition, field studies will also allow identification of malarial antigens and/or their epitopes that cause immune suppression. There are clear examples of *P. falciparum*-induced immunosuppression in patients with high parasitemia and cerebral malaria (14, 15). Even though most of the studies of immune suppression have been conducted among older subjects, similar events may be operational in infants, who are at the highest risk of dying from malaria.

In conclusion, we have identified seven naturally immunogenic T-cell determinants of AMA-1 of *P. falciparum*. Similar field-based studies are required to characterize antibody responses to the molecule. These studies will further our understanding of the nature of naturally acquired immunity against malarial infection and clinical illness and identify immunogenic regions as constituents for a potential subunit vaccine.

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