Characterization of Antibodies to Sporozoites in *Plasmodium falciparum* Malaria and Correlation with Protection

H. K. WEBSTER,[1]†* A. E. BROWN,[1] C. CHUENCHITRA,[2] B. PERMPANICH,[1] AND J. PIPITHKUL[2]

U.S. Army Medical Component[1] and Royal Thai Medical Component,[2] Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

The antibody response to sporozoites of *Plasmodium falciparum* and the role of these antibodies in protection against malaria have not been systematically investigated. An understanding of antisporozoite antibodies in natural infection is, however, important to the development of a human malaria vaccine. In a prospective study in Thailand, an antibody response to sporozoites was observed only in individuals who developed parasitemia. Antibodies were detected against an epitope in the repeat region of the circumsporozoite (CS) protein. Current candidate sporozoite vaccines are based on CS repeat antigens. The CS antibody response was of low magnitude, peaked after detection of parasitemia, and had a serum half-life of less than 1 month. CS antibody boosting occurred in only 6% of reinfected individuals. These observations suggest that antisporozoite antibody is poorly developed under natural conditions and appears not to protect against development of malaria.

Sporozoite forms of the malaria parasite—injected by the bite of an anopheline mosquito—are the target of current efforts to produce a human malaria vaccine (14, 16). Two candidate subunit vaccines have already reached the stage of preliminary human trials (2, 10). The peptide constructs of both vaccines were derived from the tetrapeptide repeat region of the circumsporozoite (CS) protein, which contains an immunodominant B-cell epitope (1, 3, 8, 21, 22). The rationale underlying development of these vaccines is that antibodies against the repeat region of the CS protein protect against sporozoite infection (13).

Despite the remarkable technological achievements in producing these candidate sporozoite vaccines, we know relatively little about the immune response to sporozoites in naturally acquired human malaria. Although antibodies against sporozoites have been identified in sera of populations living where malaria is endemic (15, 17), it is not known whether these antibodies have a protective role. Several recent studies have reported prevalence data on CS antibody in diverse geographical malarious areas (5, 6, 13); however, cross-sectional studies cannot predict whether antibodies are protective. Recently, we reported the occurrence of a CS antibody response in naturally infected Thai migrant workers (19). That study, however, was done on patients with established malaria infection and did not show antibody kinetics during the incubation period. Assessment of CS antibody function requires that antibodies be measured at the time when sporozoite challenge occurs. Furthermore, characterization of the antibody response to sporozoites in naturally acquired infections, particularly secondary or boosting responses, provides an essential view of T- and B-cell interactions with implications for human vaccine design and development.

To address the issue of a protective antisporozoite antibody response in *Plasmodium falciparum* malaria, we conducted a prospective seroepidemiological study of Thai soldiers who operate along the Thai-Laotian and -Kampu-

chean borders. Our objectives were (i) to characterize the antibody response to sporozoites of *P. falciparum* under conditions of natural exposure, (ii) to determine whether there was boosting of antibody levels on repeated exposure, and (iii) to correlate the antibody response with protection against development of parasitemia and clinical disease.

MATERIALS AND METHODS

Patient population and location. One hundred thirty-five Thai Rangers (males, aged 18 to 35 years) consented to participate in a seroepidemiological study of malaria. While at basecamp in a malaria-free area in central Thailand, all were documented to be malaria thick film negative. Two groups were defined on the basis of the following three criteria: (i) malaria history, (ii) *P. falciparum* sporozoite immunoglobulin G (IgG) antibody level, and (iii) *P. falciparum* blood stage IgG antibody level. "Malaria-experienced" subjects were positive by at least one criterion, and malaria-naive subjects were negative by all three. From base camp, the Rangers were followed to their deployment site in a range of mountains in Ubon, on the Thai border with Laos and Kampuchea. Falciparum malaria is endemic in that mountainous area, and the deployment, from August through December of 1986, coincided with the late rainy season and peak *P. falciparum* transmission.

Study design. To determine complete antibody response curves, blood was drawn for sera and malaria thick films every 10 days during the first 2 months of the study. Any Ranger who developed falciparum malaria was admitted for 7 days of supervised treatment with quinine (600 mg three times daily) and tetracycline (500 mg three times daily). Blood films were repeated daily in the unit, and sera were collected on days 0, 3, and 6. After discharge, follow-up sera and thick films were collected in the field on days 12, 18, 27, 36, 48, 57, and 77. All sera were stored on dry ice until transferred to a −70°C freezer in Bangkok.

Detection of antibodies to CS protein by ELISA. An enzyme-linked immunosorbent assay (ELISA) previously described (19) was used to detect CS antibodies against the peptide construct R32tet32. R32tet32 was produced in *Escherichia coli* and has the amino acid sequence MDP $(NANP)_{15}NVDP(NANP)_{15}NVDPtet32$, where M is methio-

* Corresponding author.

† Address for correspondence: Department of Immunology, U.S. Army Medical Component, AFRIMS, APO San Francisco, CA 96346-5000.

TABLE 1. Malaria antibody levels in Thai Rangers at the start of the study (base line) and peak responses detected during each *P. falciparum* infection

Group	Base line		Infection 1		Infection 2	
	Mean ± SEM anti-CS OD_{414} (no.)	Anti-ABS reciprocal titer (no.)	Mean ± SEM anti-CS OD_{414} (no.)	Anti-ABS reciprocal titer (no.)	Mean ± SEM anti-CS OD_{414} (no.)	Anti-ABS reciprocal titer (no.)
Malaria naive	0.02 ± 0.00 (64)	1.6 (64)	0.45 ± 0.11 (25)	175 (24)	0.22 ± 0.06 (7)	525 (7)
Malaria experienced	0.08 ± 0.03 (71)	5.5[a] (71)	0.59 ± 0.13 (28)	656[a] (27)	0.55 ± 0.19 (9)	746 (9)
Total	0.06 ± 0.01 (135)	3 (135)	0.52 ± 0.09 (53)	341 (51)	0.42 ± 0.11 (16)	640 (16)

[a] Significant difference between experienced and naive groups ($P < 0.002$).

nine, D is aspartic acid, P is proline, N is asparagine, A is alanine, V is valine, and tet32 is the first 32 amino acids encoded by a tetracycline resistance gene read out of frame (21). In brief, 50 μl of a buffered R32tet32 solution was added to wells of microtiter plates (Dynatech Laboratories, Inc.). The same solution without R32tet32 was added to wells in alternate columns. After overnight incubation, wells were

aspirated and filled with a casein blocking buffer for 1 h. Sera were diluted 1:100 in blocking buffer and added to three positive and three negative wells. After 2 h of incubation, wells were aspirated and washed twice. Peroxidase-conjugated rabbit anti-human IgG (diluted 1:2,000) was added and incubated for 1 h. Wells were then aspirated and washed three times. Peroxidase substrate was added, and 1 h later
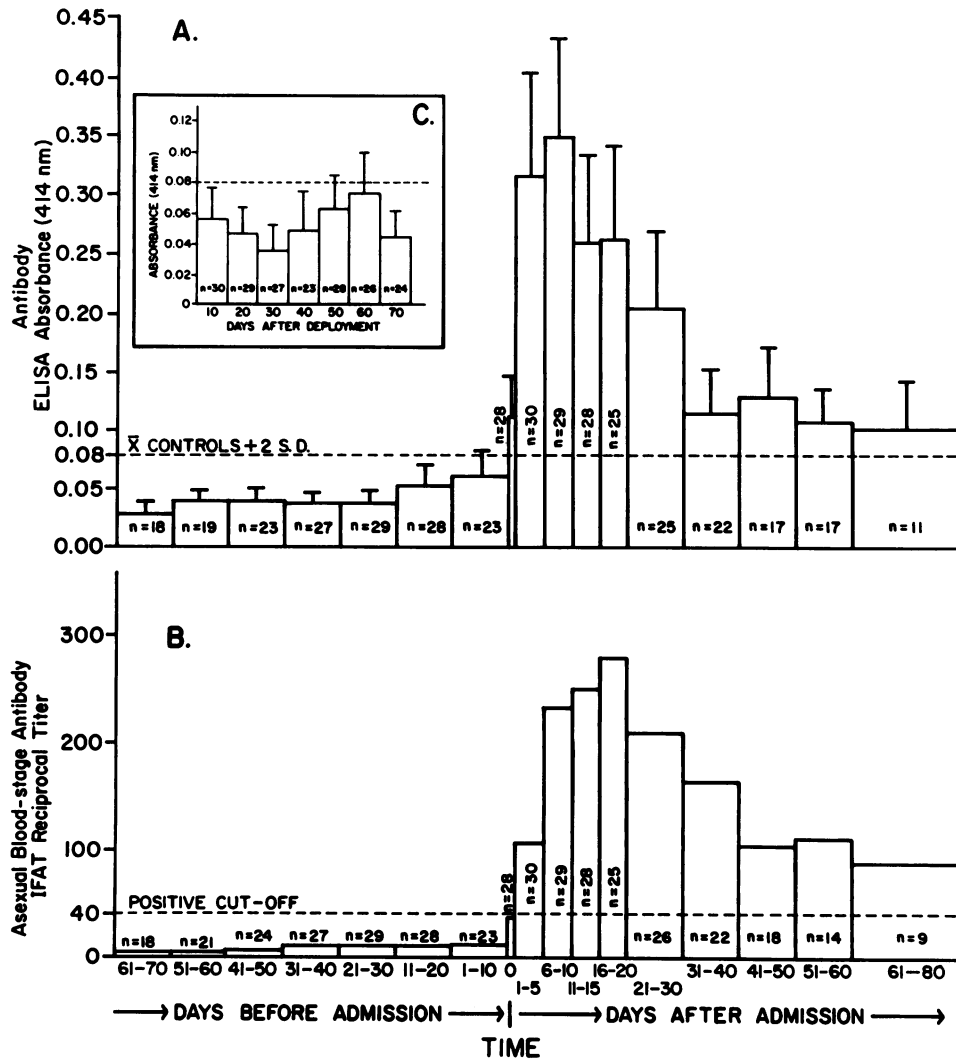

FIG. 1. Antibody response curves in 30 Thai Rangers who developed falciparum malaria. The abscissa was adjusted so that day zero is the first day of antimalarial treatment. (A) CS antibodies measured by ELISA with R32tet32 as the capture antigen. Bars represent $\bar{x}$ ± the standard error of the mean) OD of sera collected during each interval. Sera were diluted 1:100. (B) ABS antibodies determined by IFAT and represented as mean reciprocal titers. (C) CS antibody levels in 30 Thai Rangers from the same target population who did not develop malaria.

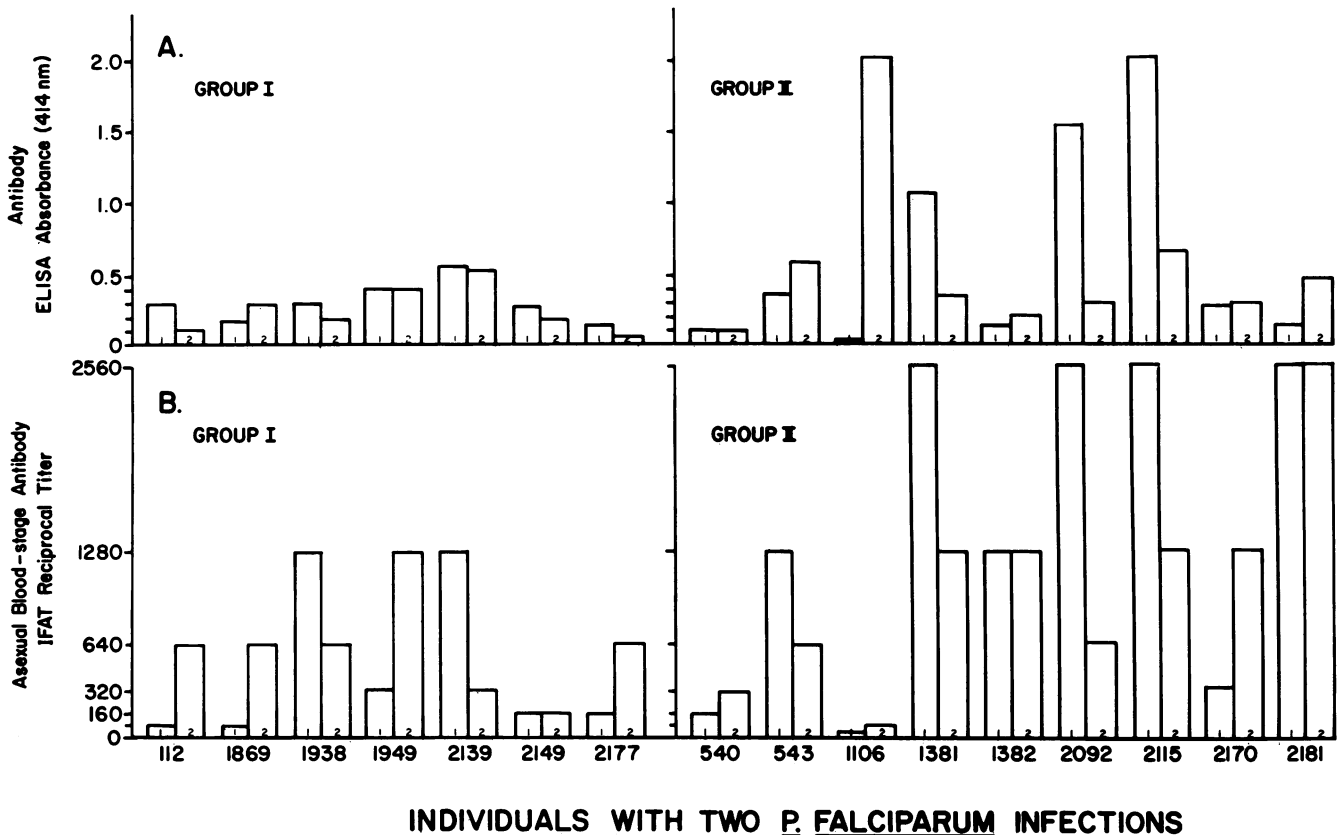INDIVIDUALS WITH TWO P. FALCIPARUM INFECTIONS

FIG. 2. Peak antibody responses to each of two clinical episodes of falciparum malaria in 16 Thai Rangers. Subject code numbers are along the abscissa, and the bars represent the pair of antibody peaks (1 and 2). Individuals are grouped on the basis of prior malaria experience (see the text). Malaria-naive subjects formed group I; malaria-experienced subjects formed group II. (A) CS antibodies measured by ELISA with R32tet32 as the capture antigen. (B) ABS antibodies determined by IFAT and represented as reciprocal titers.

the optical density (OD) at 414 nm was determined. The mean of the three negative controls was subtracted from each value, and the mean was calculated and reported as OD units. Known positive and negative control sera were assayed in parallel with each run.

Data from a negative control group were used to determine background reactivity in the ELISA and to establish a cutoff value for a positive antibody (anti-R32tet32) response. The control group consisted of 100 basic trainees (males, aged 18 to 22 years) with no history of malaria infection. A value of 0.08 OD unit ($\bar{x}$ + 2 standard deviations) was taken as the cutoff.

Detection of antibodies to the asexual blood stage (ABS) by IFAT. The indirect fluorescent-antibody test (IFAT) was based on a previously described method (19). Malaria-infected erythrocytes for thick smears were prepared from cultures of a Thai *P. falciparum* isolate. Sera were tested in twofold dilutions to the endpoint in parallel with positive and negative controls. The second antibody was goat anti-human IgG (Sigma Chemical Co., St. Louis, Mo.) conjugated to fluorescein. A reciprocal titer of 40 or greater was considered positive for the IFAT.

Statistical analysis. Two-tailed t tests of paired and independent samples were used to compare sample means.

RESULTS

Prerisk immune status. The study population was divided about evenly into malaria-naive (n = 64) and malaria-expe-

rienced (n = 71) individuals. At the start of the study, there was only marginal evidence for positive levels of CS antibodies in the malaria-experienced group (Table 1). The mean value (0.08 OD unit) of this group fell on the test cutoff (0.08 OD unit) for reactivity. For the 18 (25%) men who had positive CS antibody levels, the median was 0.12 (range, 0.09 to 1.58) OD unit.

Antibody response to natural sporozoite exposure. During the study period, 60% of the men were infected at least once with *P. falciparum*. A CS antibody response was observed only in the *P. falciparum*-infected group. This is illustrated in Fig. 1A, which was constructed from the complete serum antibody profiles of 30 Rangers. Within the group, a positive antibody response was first detected on admission to hospital, although there appeared to be a slight rise during the preceding 10 days. CS antibodies peaked during the 10 days after admission and treatment and then rapidly declined. Antibody levels at half of the peak value were observed between 21 and 30 days, suggesting a serum half-life of less than 1 month.

There were no observed differences in the CS antibody response curves between the malaria-experienced and malaria-naive groups (time to peak, 6 to 10 days; for the peak magnitude, see Table 1). Thus, primary and secondary IgG responses to CS antigen were not distinguishable. It was also apparent that preinfection CS antibody levels in the malaria-experienced group were not associated with protection since

926   WEBSTER ET AL.

the same proportions of malaria-experienced (28 of 71) and malaria-naive (25 of 64) men were infected.

A consistent CS antibody response was not observed in 30 individuals who remained aparasitemic during the study period (Fig. 1C). This observation suggested that a sporozoite inoculum sufficient to trigger a detectable immune response was invariably capable of inducing parasitemia. The observation was surprising since the 30 uninfected men and the *P. falciparum*-infected group were at equal risk.

Although most of the infected individuals showed a detectable CS antibody response, there were several hyporesponders. Of the 30 men infected for whom complete curves were available (Fig. 1A), there were 5 individuals (3 with histories of previous malaria infection) who did not produce a positive CS antibody response. Whether these hyporesponders reflect the operation of genetic factors in restricting the immune response to sporozoite epitopes remains to be demonstrated.

We also measured antibodies against the ABS (anti-ABS) of *P. falciparum*. A positive ABS antibody response was detected on admission to hospital (Fig. 1B). ABS antibodies peaked at 8 to 18 days and then declined. Individuals with malaria experience showed significantly higher ABS titers on infection than did the malaria-naive group (Table 1). The evidence for a secondary response to blood stage antigens contrasts with what we observed for CS antigen. One reason for this distinction may be that the ABS antibodies are directed against multiple epitopes, whereas the CS antibodies are measured against a repeated epitope.

Antibody boosting in natural infections. There were 16 men who were reinfected with *P. falciparum* during the study period. The average time between the first and second *P. falciparum* infections was 48 days (range, 24 to 85 days). After radical treatment, a naturally acquired subsequent infection would be expected to show CS antibody boosting. However, only 1 of 16 men demonstrated a boosting response (Fig. 2A). Antibody boosting is defined as a secondary response in which there is at least a fourfold rise in antibody compared with a previously documented response. ABS antibody boosting was also inconsistent. There were only 5 of 16 men who had a fourfold higher IFAT peak titer on reinfection (Fig. 2B). Four of the ABS-boosting responses were in men whose only prior malaria infection occurred during the study.

It was also apparent that the levels of CS antibody produced as a result of the first infection were not protective against subsequent infection. The mean CS antibody level in the 16 men reinfected was 0.11 OD unit (range, 0.02 to 0.42 OD unit; the value for each individual was taken from the sample point just before malaria diagnosis). This level of CS antibody was 2.3-fold higher than that observed before this group's first *P. falciparum* infection ($P = 0.03$). After reinfection, the mean peak CS antibody level was 0.42 OD unit (Table 1). These antibody levels did not protect against development of parasitemia.

DISCUSSION

An antibody response to the immunodominant epitope of the CS protein of *P. falciparum* was observed in 83% of Thai Rangers with naturally acquired infections. Using a prospective study design, we observed that the peak antibody response occurred during a 10-day interval after diagnosis of patent parasitemia. A slight rise in CS antibody level was observed in the 10 days just before detection of parasitemia. Sixteen percent of parasitemic individuals had no detectable

CS antibody response. In responding individuals, the magnitude of the antibody response was not influenced by previous malaria experience. The serum antibody (IgG) half-life was less than 1 month. A boosting CS antibody response was observed in only 6% of individuals reinfected with *P. falciparum*. Previous malaria experience or recent malaria infection in this study population was not associated with protective levels of CS antibodies.

CS antibodies may not be sufficient to confer protection against erythrocytic infection in the host. Antibodies against the nonrepeat regions of the CS protein or antibodies against other antigenic components of the sporozoite surface remain obscure in terms of any protective specificities. Cell-mediated immunity may, however, contribute to protection against sporozoite infection. Studies with animals have shown protection against sporozoites to involve cell-mediated responses (7). However, studies with mice with defined *H-2* haplotypes have shown genetic restriction in antibody and cellular responses to the T-cell epitope(s) of peptide contructs of the CS repeat region (4, 9, 18). Whether these observations are characteristic of the human immune responses to sporozoites is not known. Our observations on CS antibody boosting, however, suggest that there are problems with T-cell function—possibly due to epitope variation or genetic restriction and possibly due to parasite-induced immunosuppressive or immunoregulatory defects in the host (11, 20). Additionally, the low sporozoite dose from a mosquito inoculation, and thus the small amount of CS antigen, may produce poor levels of lymphocyte priming and induction of memory cells. Development of a high-titer CS antibody response probably depends on frequent mosquito inoculation of sporozoites over a long period of time (years). Occupationally exposed groups like the Thai Rangers in this study appear not to develop protective immunity against sporozoite infection.

Evidence against a protective role for CS antibodies in natural *P. falciparum* infections was also obtained in a prospective study done in Kenya (12). This study, which followed up adults after curative treatment, found that CS antibody levels in individuals subsequently infected were indistinguishable from those of individuals who did not develop parasitemia. There was also no apparent relationship between day of onset of parasitemia and level of CS antibody.

For a sporozoite vaccine to be effective, it will have to be sufficiently immunogenic to induce antibody levels greater than those observed in this study. Furthermore, high antibody levels must be maintained in the host so that they function at the time of sporozoite inoculation—a condition that would depend on natural boosting by sporozoites or repetitive administration of a vaccine. However, the studies reported here in falciparum malaria clearly suggest that boosting of CS antibodies is poorly developed and that the levels of these antibodies may not be sufficient to confer protection in natural infection.

Although CS antibodies appear not to protect against parasitemia, they may function to modify the severity of infection by reducing the number of sporozoites which successfully invade liver cells. This may be one way of avoiding superinfection, which would be destructive to both the host and the parasite. Additionally, CS antibodies, because of their short half-life, provide an epidemiological marker for measuring active transmission in malaria-exposed populations.

LITERATURE CITED

1. Ballou, W. R., J. Rothbard, R. A. Wirtz, D. M. Gordon, J. S. Williams, R. W. Gore, I. Schneider, M. R. Hollingdale, R. L. Beaudoin, W. L. Maloy, L. H. Miller, and W. T. Hockmeyer. 1985. Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum*. Science 228:996–999.

2. Ballou, W. R., J. A. Sherwood, F. A. Neva, D. M. Gordon, R. A. Wirtz, G. F. Wasserman, C. L. Diggs, S. L. Hoffman, M. R. Hollingdale, W. T. Hockmeyer, I. Schneider, J. F. Young, P. Reeve, and J. D. Chulay. 1987. Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. Lancet i:1277–1281.

3. Dame, J. B., J. S. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, G. S. Sanders, E. P. Reddy, C. L. Diggs, and L. H. Miller. 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science 225: 593–599.

4. Del Giudice, G., J. A. Cooper, J. Merino, A. S. Verdini, A. Pessi, A. R. Togna, H. D. Engers, G. Corradin, and P.-H. Lambert. 1986. The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is I-A^b-restricted: possible implications for malaria vaccines. J. Immunol. 137:2952–2955.

5. Del Giudice, G., H. D. Engers, C. Tougne, S. S. Biro, N. Weiss, A. S. Verdini, A. Pessi, A. A. Degremont, T. A. Freyvogel, P.-H. Lambert, and M. Tanner. 1987. Antibodies to the repetitive epitope of *Plasmodium falciparum* circumsporozoite protein in a rural Tanzanian community: a longitudinal study of 132 children. Am. J. Trop. Med. Hyg. 36:203–212.

6. Del Guidice, G., A. S. Verdini, M. Pinori, A. Pessi, J.-P. Verhave, C. Tougne, B. Ivanoff, P.-H. Lambert, and H. D. Engers. 1987. Detection of human antibodies against *Plasmodium falciparum* sporozoites using synthetic peptides. J. Clin. Microbiol. 25:91–96.

7. Egan, J. E., J. L. Weber, W. R. Ballou, M. R. Hollingdale, W. R. Majarian, D. M. Gordon, W. L. Maloy, S. L. Hoffman, R. A. Wirtz, I. Schneider, G. R. Woollett, J. F. Young, and W. T. Hockmeyer. 1987. Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. Science 236:453–456.

8. Enea, V., J. Ellis, F. Zavala, D. E. Arnot, A. Asavanich, A. Masuda, I. Quakyi, and R. S. Nussenzweig. 1984. DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino acid sequence of repetitive epitope. Science 225:628–630.

9. Good, M. F., J. A. Berzofsky, W. L. Maloy, Y. Hayashi, N. Fujii, W. T. Hockmeyer, and L. H. Miller. 1986. Genetic control of the immune response in mice to a *Plasmodium falciparum* sporozoite vaccine. J. Exp. Med. 164:655–660.

10. Herrington, D. A., D. F. Clyde, G. Losonsky, M. Cortesia, J. R. Murphy, J. Davis, S. Baqar, A. M. Felix, E. P. Heimer, D. Gillessen, E. Nardin, R. S. Nussenzweig, V. Nussenzweig, M. R. Hollingdale, and M. M. Levine. 1987. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Nature 328:257–259.

11. Ho, M., H. K. Webster, S. Looareesuwan, W. Supanaranond, R. E. Phillips, P. Chanthavanich, and D. A. Warrell. 1986. Antigen-specific immunosuppression in human malaria due to *Plasmodium falciparum*. J. Infect. Dis. 153:763–771.

12. Hoffman, S. L., C. N. Oster, C. V. Plowe, G. R. Woollett, J. C. Beier, J. D. Chulay, R. A. Wirtz, M. R. Hollingdale, and M. Mugambi. 1987. Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. Science 237:639–642.

13. Hoffman, S. L., R. Wistar, W. R. Ballou, M. R. Hollingdale, R. A. Wirtz, I. Schneider, H. A. Marwoto, and W. T. Hockmeyer. 1986. Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of *Plasmodium falciparum*. N. Engl. J. Med. 315:601–606.

14. Miller, L. H., R. J. Howard, R. Carter, M. F. Good, V. Nussenzweig, and R. S Nussenzweig. 1986. Research toward malaria vaccines. Science 234:1349–1356.

15. Nardin, E. H., R. S. Nussenzweig, I. A. McGregor, and J. H. Bryan. 1979. Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. Nature (London) 206:597–599.

16. Nussenzweig, V., and R. S. Nussenzweig. 1986. Development of a sporozoite malaria vaccine. Am. J. Trop. Med. Hyg. 35: 678–688.

17. Tapchaisri, P., Y. Chomcharn, C. Poonthong, A. Asavanich, S. Limsuwan, O. Maleevan, S. Tharvanij, and T. Harinsuta. 1983. Anti-sporozoite antibodies induced by natural infection. Am. J. Trop. Med. Hyg. 32:1203–1208.

18. Togna, A. R., G. Del Guidice, A. S. Verdini, F. Bonelli, A. Pessi, H. D. Engers, and G. Corradin. 1986. Synthetic *Plasmodium falciparum* circumsporozoite peptides elicit heterogenous L3T4$^+$ T cell proliferative responses in *H-2^b* mice. J. Immunol. 137:2956–2960.

19. Webster, H. K., E. F. Boudreau, L. W. Pang, B. Permpanich, P. Sookto, and R. A. Wirtz. 1987. Development of immunity in natural *Plasmodium falciparum* malaria: antibodies to the falciparum sporozoite vaccine 1 antigen (R32tet32). J. Clin. Microbiol. 25:1002–1008.

20. Webster, H. K., M. Ho, S. Looareesuwan, K. Pavanand, Y. Wattanagoon, D. A. Warrell, and W. T. Hockmeyer. 1988. Lymphocyte responsiveness to a candidate malaria sporozoite vaccine (R32tet32) of individuals with naturally acquired *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg. 38:37–41.

21. Young, J. F., W. T. Hockmeyer, M. Gross, W. R. Ballou, R. A. Wirtz, J. H. Trosper, R. L. Beaudoin, M. R. Hollingdale, L. H. Miller, C. L. Diggs, and M. Rosenberg. 1985. Expression of *Plasmodium falciparum* circumsporozoite protein in *E. coli* for potential use in a human malaria vaccine. Science 228:958–962.

22. Zavala, F., J. P. Tam, A. H. Cochrane, I. Quakyi, R. S. Nussenzweig, and V. Nussenzweig. 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. Science 228:1436–1440.