Development of Immunity in Natural *Plasmodium falciparum* Malaria: Antibodies to the Falciparum Sporozoite Vaccine 1 Antigen (R32tet32)

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Antibodies that reacted with a candidate sporozoite vaccine antigen (R32tet32) were found in 20 of 21 patients treated for acute infection with *Plasmodium falciparum* and monitored longitudinally over 67 days. R32tet32 contains 32 tandem copies of a tetrapeptide sequence that constitutes the immunodominant epitope of the circumsporozoite surface protein. The magnitude of the antibody response varied considerably among individuals and appeared to be independent of the number of previous clinical infections. Recrudescence of infection or infection with *Plasmodium vivax* had no demonstrable effect on antibody levels, although reinfection with *P. falciparum* produced a rapid rise in antibody titer. Antibody levels were observed to decline rapidly after treatment of clinical infection with mefloquine. The apparent antibody half-life was 27 days, which is comparable to the half-life of circulating immunoglobulin G in humans. The data suggest that antisporeozoite antibody production ceased on about day 4 after treatment of acute infection. A similar pattern of response was observed for antibodies against the erythrocytic forms of the parasite. The cessation of antibody synthesis was interpreted as being due to immunosuppression induced by the presence of intraerythrocytic parasites and may explain in part why protective immunity is poorly developed in natural malaria infections.

Natural malaria infection develops after injection of sporozoites into the host by an anopheline mosquito during a blood meal. Although the sporozoites rapidly leave the circulation by invading host liver cells or through generalized phagocytosis, a humoral response to the sporozoites does develop (14, 22, 23). The response involves both sporozoite-precipitating and -neutralizing antibodies which act both in vivo (15) and in vitro (11) to impair penetration into or development of the vector stage in host hepatocytes or both. Under experimental conditions, protective immunity to sporozoite challenge has been demonstrated in rodents, monkeys, and humans (3, 16–18), although there have been only limited studies with humans. Whether protective antisporeozoite immunity occurs under natural conditions of infection with human malaria is not known. However, based on studies with the erythrocytic stages of malaria, it is generally appreciated that immunity to natural malaria is slow to develop, poorly protective, and readily lost on leaving an endemic area (13).

Studies using monoclonal antibodies have shown that the target antigen of the immune response is a major polypeptide covering the sporozoite surface, the circumsporozoite (CS) protein (21, 30). After pioneering work with the simian parasite *Plasmodium knowlesi* (7), the gene encoding the CS protein in *Plasmodium falciparum* (5) was cloned and sequenced and the amino acid structure was deduced. The CS protein of *P. falciparum* contains a tetrapeptide sequence repeated 41 times, constituting a central core region containing the immunodominant epitope that is the primary target of antisporeozoite immunity. Both recombinant (29) and synthetic (31) products representing sequences of the immunodominant region of the CS protein have been produced as candidate vaccines.

Falciparum sporozoite vaccine 1 contains as the antigen R32tet32, which is a recombinant gene product produced in *Escherichia coli* as a candidate human malaria vaccine (29). R32tet32 is a construct of 32 tandem copies of a CS tetrapeptide repeating sequence, [(Asn-Ala-Asn-Pro)5-(Asn-Val-Asp-Pro)], fused to 32 amino acids derived from an open reading frame in the Te region of the pASI *E. coli* expression plasmid. R32tet32 must contain both B- and T-cell epitopes to be an effective vaccine. In mice, R32tet32 produces high titers of antibody that react specifically with CS protein on the surface of live sporozoites (positive circumsporozoite precipitin [CSP] reaction) and inhibits sporozoite infection of human hepatoma cells (1, 29). At present, falciparum sporozoite vaccine 1 is undergoing phase I clinical trials in the United States.

We report here on the occurrence of antibodies to R32tet32 and their quantitative and temporal characteristics in plasma from individuals naturally infected with *P. falciparum* malaria. Our observations suggest that antisporeozoite antibodies were not protective against naturally occurring malaria in Thailand. It further appears that immunosuppression associated with acute infection acted to prevent development of an optimal immune response.

**MATERIALS AND METHODS**

**Subjects.** Plasma samples were collected from 21 patients with diagnosed *P. falciparum* malaria and 8 healthy controls. The patients and controls were participants in a pharmaco-
kinetic study on mefloquine treatment of acute falciparum malaria (E. F. Boudreau, L. W. Pang, A. C. Schroeder, G. E. Childs, and L. Fleckstein, unpublished study). The subjects were migrant workers employed in fruit and rubber plantations in Trat province in southeastern Thailand. All patients were male (ages, 19 to 40 years) and had no detectable intercurrent diseases. The controls (males; ages, 24 to 31 years) were from the same area as the patients. They were essentially healthy and had negative peripheral blood smears for malaria parasites. Two individuals reported a history of malaria infection during the previous 5 years. Samples were collected on days 0, 4, 11, 18, 25, 28, 32, 39, 53, and 67 in accordance with the mefloquine study protocol. None of the subjects had taken any antimalarial medications for 1 week before entering the study. After the day 0 blood samples were drawn, the patients were treated (a single 750- or 1,500-mg dose of mefloquine) for malaria, and all subsequent samples were posttreatment. There were 10 patients that received 1,500 mg of mefloquine (patients 1, 4, 6, 8, 9, 14, 15, 16, 19, and 20) and 11 patients that received a 750 mg-dose (patients 2, 3, 5, 7, 10, 11, 12, 13, 17, 18, and 21) (Table 1). Mefloquine doses were equally divided among the eight controls. Individuals whose infection recrudesced were treated with quinine and tetracycline. Heparinized blood samples were separated immediately into plasma and erythrocytes, and the plasma was stored when collected at −20°C and later in the laboratory at −70°C. Patients were kept in the hospital until aparasitemic, discharged, and monitored for 67 days.

A second group of controls consisted of 86 individuals from a nonmalarious area with no history of clinical malaria infection or antimalarial drug use. All individuals were male (ages, 18 to 31 years) and essentially healthy. A single blood sample was obtained from each of the controls.

Detection of antibodies to R32tet32 by ELISA. Anti-R32tet32 antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) by using R32tet32 (29) as the capture antigen in microtiter plates (Immunol 2 "U"; Dynatech Laboratories, Inc., Alexandria, Va.). Stock R32tet32 was diluted to a working concentration of 2 μg/ml of phosphate-buffered saline (PBS) containing bovine casein (4 μg/ml), and 50 μl was added to each of the wells in the even-numbered columns. PBS-boiled casein without antigen was added to wells in the odd-numbered columns. The plates were covered and incubated overnight. The contents of the wells were then aspirated, and the wells were filled with blocking buffer (0.5% bovine casein, 0.005% thimerosal, 0.001% phenol red) containing 1% Tween 20 (TW) and held for 1 h. The plasma to be tested was diluted 1:100 in blocking buffer containing 0.025% TW, and 50 μl was added to each of three adjacent even- and odd-numbered-column wells and incubated for 2 h. The peripheral wells were not used. The contents of the wells were then aspirated and washed twice with PBS containing 0.05% TW, and 50 μl of rabbit anti-human immunoglobulin G (IgG) (H+L chains)-peroxidase conjugate (Miles-Yeda Ltd., Naperville, Ill.) diluted 1:2,000 in blocking buffer containing 0.025% TW was added to each well. After a 1-h incubation, the contents of the wells were aspirated. The wells were washed three times with PBS-TW, 100 μl of peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added per well, and the A414 (expressed as absorbance units [AU]) was recorded after 1 h. All incubations were carried out at room temperature. The mean negative control value (three wells without R32tet32) was subtracted from the absorbance values for wells containing R32tet32, and a mean value was calculated. Standard negative and positive control sera were assayed in each plate.

IFAT for detection of antibodies to the IE stages of P. falciparum. The indirect fluorescent-antibody test (IFAT) for detection of antibodies to the intraerythrocytic (IE) stages of P. falciparum was based on a method described previously (26). Malaria antigen for thick smears was prepared from an isolate of P. falciparum collected in southeastern Thailand. Plasma samples were tested in twofold dilutions from 1:10 to 1:1,280, and positive, negative, and PBS controls were incorporated in each sample set. The second antibody was goat anti-human IgG conjugated to fluorescein (Sigma Chemical Co., St. Louis, Mo.). A sample was considered positive when it was reactive at a dilution of 1:40.

Statistical analysis. The Student t test (two tailed) was used to determine the significance of differences between means. IFAT data were log transformed to compute the geometric mean reciprocal titer. The log values were converted back and are reported as reciprocal titers.

RESULTS

The characteristics of the malaria patients and healthy controls are shown in Table 1. There were 21 adult male patients, all of whom reported one or more previous symptomatic malaria infections (average, 3 to 4 cases). The arithmetic mean parasite count on admission was 46,866 ± 6,415/μl of blood. All patients were treated with mefloquine and monitored for 67 days. The average time in the hospital before discharge was 4 days. Ten individuals were cured of infection without further complications during the study period. Two patients were cured of P. falciparum but experienced Plasmodium vivax infection on about day 35. There were seven patients with infections that recrudesced (average, 23 days), one of whom subsequently also developed P. vivax infection on day 66. Two patients were considered to have reinfections on days 66 and 67. All patients had positive IFA titers on admission, and all but one had maximum ELISA titers greater than the mean control value.

The antibody disappearance curves for 10 patients with uncomplicated malaria who were sampled 10 times over 67 days are shown in Fig. 1. Natural sporozoite infection was estimated to have occurred in these patients 10 to 14 days before presentation at the clinic (on the average, a person was symptomatic for about 3 days before seeking treatment). It is not known, however, whether any of the patients were parasitic but asymptomatic before their clinical episode. Among the 10 uncomplicated cases, the range in maximum antibody levels to R32tet32 was considerable (0.045 to 2.000 AU). In the 10 patients with uncomplicated malaria, the maximum antibody response was recorded on day 0 for 6 patients and on day 4 for 3 patients. One patient did not have a detectable antibody response. A steep decline in antibody level was apparent in most patients, with an average half-maximum absorbance occurring at 27 days. By day 67, the mean absorbance for all 10 patients was 0.158 AU, which was fivefold less than the maximum absorbance of 0.81 AU.

The anti-R32tet32 ELISA curves for 11 patients in whom outcomes were complicated (seven recrudescences, two reinfections with P. falciparum, and three P. vivax infections) are shown in Fig. 2 (see also Table 1). Overall, the ranges of antibody levels and shapes of the curves were similar to those in Fig. 1. The mean half-maximum absorbance for this group was at 25 days. Recrudescence of
infection or infection with *P. vivax* appeared not to influence the levels of anti-R32tet32 antibodies. In one patient (no. 14, Fig. 2), parasites were detected on day 67 and there was a sharp rise in antibody level that began after day 53, indicating reinfection with *P. falciparum*. Another patient (no. 19) did not show a change in antibody titer, although parasites were observed on day 66.

The anti-R32tet32 ELISA curves for all 21 patients and the 8 controls are shown in Fig. 3. For the patient group, the mean maximum antibody level occurred on day 4 (X, 0.928 AU; 95% confidence interval [CI], 0.637 to 1.220), with the half-maximum absorbance occurring on day 27. The eight controls had a mean maximum absorbance over the 67-day period of 0.039 AU (95% CI, −0.015 to 0.093). For comparison, a group of 86 individuals, residing in a malaria-free region, with no history of clinical malaria infection had a mean anti-R32tet32 ELISA titer of 0.040 AU (95% CI, 0.027 to 0.052).

The geometric mean levels of IE-stage antibodies for the group of 21 *P. falciparum*-infected patients are shown in Fig. 4. The maximum geometric mean titer for the eight controls over the 67-day period was 3 (95% CI, 0.6 to 11). The IFAT titer for the malaria-infected group peaked on day 11 (geometric mean, 661; 95% CI, 495 to 884), although there was no statistical difference between the titers on days 4 and 11, and then decreased over the next 21 days. The decrease in the IFAT titer was significant (*P* < 0.005) by day 28. Over the next 36 days, the titer decreased slightly but appeared to level off. The calculated half-maximum titer, 330, for the anti-IE-stage antibodies occurred on day 34. All individuals except one had reactive IFAT titers (≥40) on day 0, suggesting previous clinical infection(s).

**DISCUSSION**

Antibodies which reacted with the R32tet32 antigen were clearly present in plasma from individuals naturally infected with *P. falciparum*. These studies provide a longitudinal profile (67 days) of anti-R32tet32 antibody levels in patients treated for symptomatic infection and monitored by sequential sampling. A previous study with randomly collected sera from The Gambia used an immunoradiometric assay to demonstrate that antiparasite antibodies were reactive with a synthetic dodecapeptide, (NANP)₃, which also contained the immunodominant epitope of the CS protein (31). This same study showed that results from the radiometric assay correlated well with an IFAT and that preincubation of sera with (NANP)₃ abolished the IFAT reaction to sporozoites.

Antiparasite antibodies were first demonstrated in natural falciparum infections in 1979 (14) by an IFAT with glutaraldehyde-fixed sporozoites; the CSP test also was used. Since then other groups (22, 23) have used the IFAT and CSP test to assess the prevalence of antiparasite
antibodies. One study in Thailand (23) which examined both IgG and IgM antibodies by IFAT found predominantly IgG in a high proportion (57%) of adults living in an endemic area. This same group noted that the mean antispoozoite antibody titer for paired serum samples from 108 patients followed up in a malaria-free area decreased significantly after 63 days (22). Persistent low titers of antispoozoite antibodies have been reported in a small number of African expatriates living in France (6).

Recently, the presence of antibodies to the R32tet32 antigen was demonstrated in sera from naturally infected individuals residing in a hyperendemic malarious area of Indonesia (10). These findings, based on prevalence data and in vitro assays, were interpreted as providing evidence for a protective role for antispoozoite antibodies in natural infections.

The individuals in the present study all had naturally acquired P. falciparum malaria. Falciparum malaria in southeastern Thailand is seasonal and associated with variable levels of mosquito transmission. The habits of migrant workers also result in daily and seasonal variation in risk. Although all individuals had antibodies to the IE stage (IFAT) of P. falciparum and all but one had antibodies to the spoozoite stage (ELISA), none had sufficient immunity to prevent symptomatic infection.

Individual anti-R32tet32 levels showed considerable variation in terms of maximum observed titers. In one patient (no. 7), there appeared to be no discernible rise in detectable antibody level. A similar picture was observed in rhesus monkeys immunized with Plasmodium cynomolgus spoozoites, in which considerable variation in CSP antibody response was noted for individual monkeys even though they were subjected to similar immunization schedules (D. Chen, Ph.D. thesis, New York University School of Medicine, New York, 1974).

The level of anti-R32tet32 antibody response appeared not to be related to the number of previous clinical infections. Recrudescence of infection or infection with P. vivax had no apparent effect on anti-R32tet32 antibody levels as observed during the period of study. This observation is consistent with the stage and species specificity of the immune response in malaria (3, 16).

Based on grouped data, the results of the study showed a maximum anti-R32tet32 antibody response on day 4 after presentation for treatment. Only 3 of 20 patients with anti-R32tet32 titers had maximum levels on day 0. On the average, an individual would have received an infective mosquito bite, hence spoozoite exposure, about 10 to 14 days before arrival at the clinic. Thus, most patients were experiencing a rising anti-R32tet32 titer at the time a blood stage infection developed that was sufficient to cause symptoms. After day 4, there was a relatively steep decline in anti-R32tet32 antibody levels. The calculated half-maximum absorbance for the group was at 27 days. A priori, there is no reason to expect such consistent behavior in antibody response among this number of individuals. In theory, the magnitude and duration of the anti-R32tet32 (spoozoite) response depends on multiple parameters: prior and subse-
quent sporozoite exposure relative to the infective bite (number, frequency, and interval of mosquito bites), sporozoite dose and viability, presence of blood stage parasites, prophylactic or other drugs, and existing levels of antiparasite immunity (humoral and cell mediated).

In fact, the antibody half-life in this group was less than 1 month. The apparent antibody half-life was estimated from the group mean half-maximum absorbance (27 days). It is of interest to consider that the half-life of circulating IgG in humans is 23 days (27). Thus, it would appear that production of anti-R32tet32 IgG ceased on about day 4 of the study period. Since these individuals would be predicted to be experiencing secondary responses, the antibody half-life appears shortened, more like a primary response pattern. Specifically, in the case of a human immunization with irradiated falciparum sporozoites to prevent clinical infection, protection was observed for up to 3 months, which correlated with CSP-detectable antibodies (2). Blood stage (IE) antibodies have been observed to persist for more than 1 year after clinical infection with *P. falciparum*, although variation and decrease in titer was noted (4, 12).

There are two major events which occur in the host at about day 4 that could effect the immune system. First, a patent IE-stage parasitemia has been developing in the host for 7 or more days. Lymphocyte responsiveness to malaria antigens has been shown to be specifically suppressed during acute falciparum infection. This includes both blood stage (9) and sporozoite (R32tet32) (H. K. Webster, M. Ho, S. Looareesuwan, K. Pavanand, Y. Wattanagoon, D. A. Warrell, and W. T. Hockmeyer, submitted for publication) antigens. This suppression is reversible, and lymphocyte responsiveness tends to be restored in most convalescent individuals by 5 weeks post-curarive treatment. At moderate-to-high parasitemias, the suppression involves lymphocyte responses to heterologous antigens (9) and the humoral response to various immunizations (28). A defect in T-helper lymphocyte function could disrupt specific B-cell activation and hence disrupt antibody synthesis. In *Plasmodium berghei*-infected mice the presence of an IE-stage infection was noted to suppress the antibody response to injected (irradiated) sporozoites (20). A second dose of sporozoites failed to elicit a secondary antibody response. However, once a CSP antibody response was established in an animal, the occurrence of IE-stage infection had no apparent effect on antibody levels.

The second major event in the host was that treatment of infection on day 0 would have resulted in peak mefloquine levels in blood within 4 h. The half-life of mefloquine in blood after a single dose (1,500 mg) is approximately 14 days (E. F. Boudreau, personal communication). Mefloquine, like many other antimalarial agents, has been shown to suppress lymphocyte function in vitro (24). So it is possible that mefloquine treatment could result in toxicity to T and B lymphocytes, thereby shutting down antibody synthesis. However, the drug effect would be predicted to be nonspecific and, therefore, should produce a generalized suppression of the immune response. Other antimalarial agents, such as chloroquine and quinine, have also been shown to suppress lymphocyte responses in vitro. However, both of these antimalarial agents had no effect on the immune response of mice in vivo (25). In fact, immunization of rodents with live sporozoites was found to produce good protective responses to subsequent challenge when IE-stage infection was prevented by concomitant administration of chloroquine (19). Clinical infection was observed to suppress the response in Nigerian children to a meningococcal polysaccharide vaccine (8). However, when children were placed on chloroquine chemoprophylaxis, the response was enhanced (8).

In the lymphocyte study (9) showing malaria antigen-specific immunosuppression, the responses to streptococcal (streptokinase-streptodornase) and tubercular (purified protein derivative) antigens were not suppressed at low-to-moderate parasitemias. The individuals in that study were treated with quinine (an aminoquinoline like mefloquine) and tetracycline.

Antibody levels (IFAT) to the IE stage of *P. falciparum* showed a profile that was similar to that observed for anti-R32tet32. The anti-IE-stage antibodies appeared to peak on day 11, and the falloff in the geometric mean titer was less precipitous. A decline in IE-stage antibodies has been observed previously (4, 12). For the seven patients whose infections recrudesced (average, 23 days), there was no evidence of a booster effect on antibody levels. The lack of antibody response is consistent with immunosuppression.
from the previous clinical parasitemia. It is also possible that a subpatent parasitemia has consequences for the immune response.

Given the observations on antibody responses in this study and evidence from other studies that lymphocyte responsiveness is suppressed during clinical infection (9; Webster et al., submitted), it is tempting to speculate that one of the reasons for poorly developed humoral immune response in natural malaria is that the response is shut down before protective levels of antibody are achieved in the host. Although this interpretation is applicable to the malaria situation in Thailand, its relevance to different epidemiological settings (e.g., Africa or Indonesia) is unknown.

The observations in this study showed that most of the naturally infected (sensitized) individuals from an endemic area produced antibodies against sporozoites that reacted with the R32et32 recombinant peptide. In these individuals the levels of antisporeozone antibodies were not protective in the sense of preventing clinical infection. They probably do play an important role in modulating the infective sporozoite dose in the host. It appeared, however, that as the infection progressed to a patent parasitemia the production of new malaria-specific IgG antibody ceased. This was interpreted in the light of other studies as being due to antigen-specific immunosuppression associated with the parasitemia. There also appeared to be a similar suppression in the development of anti-IE-stage antibodies. However, a direct or concomitant effect on antibody production due to mefloquine treatment, although considered negligible, cannot be ruled out.

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