

AD-A232 196

REPORT DOCUMENTATION PAGE

Unclassified		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 90-118		7a. NAME OF MONITORING ORGANIZATION Naval Medical Command	
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research	6b. OFFICE SYMBOL (If applicable)	7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120	
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command	8b. OFFICE SYMBOL (If applicable)	10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		PROGRAM ELEMENT NO. 63807A	PROJECT NO. 3M462907.D808
		TASK NO. AQ133	WORK UNIT ACCESSION NO DA301569
11. TITLE (Include Security Classification) Plasmodium falciparum-infected anopheles stephensi inconsistently transmit malaria to humans			
12. PERSONAL AUTHOR(S) Rickman LS, Jones TR, Long GW, Paparello S, Schneider I, Paul CF, Beaudoin RL, Hoffman SL			
13a. TYPE OF REPORT journal article	13b. TIME COVERED FROM TO	14. DATE OF REPORT (Year, Month, Day) 1990	15. PAGE COUNT 5
16. SUPPLEMENTARY NOTATION Reprinted from: American Journal of Tropical Medicine and Hygiene 1990 Vol.43(5) pp. 90-120			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
		Plasmodium falciparum, human malaria, anopheles stephensi, sporozoites, human volunteers, infectivity, gametocyte cultures, NF54 strain, 3D7 clone	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
A-1 20			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division		22b. TELEPHONE (Include Area Code) 202-295-2188	22c. OFFICE SYMBOL ISD/ADMIN/NMRI

PLASMODIUM FALCIPARUM-INFECTED ANOPHELES STEPHENSII INCONSISTENTLY TRANSMIT MALARIA TO HUMANS

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Abstract. Malaria was transmitted to only 5 of 10 volunteers bitten by 1-2 *Anopheles stephensi* carrying sporozoites of the 3D7 clone of the NF54 strain of *Plasmodium falciparum* in their salivary glands. Parasites were detectable by culture in blood taken 7-10 days following exposure and by thick blood film 14-16.5 days after exposure. Infectivity did not correlate with the numbers of sporozoites in the salivary glands.

Studies which preceded the first trials of malaria sporozoite vaccines showed that the bites of 5 *Anopheles stephensi* infected with *Plasmodium falciparum* reproducibly infected volunteers.^{1,2} When immunized volunteers were bitten by 5 infected mosquitoes within 1 hr, there was a significant delay in the onset of parasitemia, but only 2 of 23 immunized volunteers did not become infected with blood stage parasites.³⁻⁷ In rodent malaria models, antibody-induced protective immunity can be overcome by increasing the sporozoite inoculum.⁸ In malaria endemic areas, some individuals are not exposed to the bite of more than 1 or 2 infected mosquitoes per night, and may never be exposed to 5 infected mosquitoes within 1 hr. Therefore, it has been suggested that the failure to show complete protection in most volunteers previously immunized with sporozoite vaccines may have been due to challenging them with an unrealistically large sporozoite inoculum.^{5,6} Earlier studies disagree on the effect of the number of infective bites upon the onset of parasitemia and the percentage of subjects ultimately infected.⁹⁻¹¹ These studies differ from current studies in 1 important aspect. In the older studies, the mosquitoes were infected by feeding upon gametocytemic humans; the mosquitoes used in our studies are infected by membrane feedings on gametocytemic blood containing parasites that have been maintained in *in vitro* culture for several years. This current study was designed to determine the fewest number of bites by infected mosquitoes required to reliably transmit *P. falciparum* of the 3D7 clone of the NF54 strain to all members of a study population so that future

vaccine trials can be designed to maximize the possibility of demonstrating protective immunity.

MATERIALS AND METHODS

Subjects

Ten adult males, 25-39 years of age, were recruited from the active duty military staff of the Naval Medical Research Institute (NMRI) and National Naval Medical Center (NNMC). The protocol was approved by institutional review committees at NMRI and NNMC, and written informed consent was obtained from each volunteer. None of the volunteers had cardiovascular, liver, or renal function abnormalities, was taking immuno-suppressive or antimalarial medications, had been infected with malaria, had antibodies to malaria sporozoites, blood stage parasites, or human immunodeficiency virus, or had hepatitis B surface antigenemia.

Parasites, mosquitoes, and infection of volunteers

The 3D7 clone of the NF54 strain of *P. falciparum*, which is sensitive to chloroquine, pyrimethamine, and quinine, was used in this study.¹² It was maintained in culture through standard methods.^{1,13} Mature gametocytes appeared in cultures 14-16 days post-inoculation. Three days after reaching adulthood, laboratory-reared *An. stephensi* were infected by membrane feeding upon gametocyte-rich cultures and were maintained during the extrinsic incubation period with dry sugar and water-soaked cotton balls. Sixteen to 17 days after the bloodmeal, the mosquitoes

were permitted to feed on the volunteers. Each volunteer received a bite from a mosquito which was subsequently dissected to demonstrate the presence of blood engorgement and sporozoites in the salivary glands. The numbers of sporozoites in the salivary glands of a mosquito were graded as follows: 1–10 = 1+, 11–100 = 2+, 101–1,000 = 3+, and > 1,000 = 4+. A mosquito was considered possibly to have delivered sporozoites if she was engorged with blood and had sporozoites in her salivary glands. In the first experiment, 5 volunteers were exposed to mosquitoes until each subject received 1 such bite; in the second experiment, 5 volunteers were exposed to mosquitoes until each subject received 2 such bites.

Diagnosis of malaria

Twice daily, beginning on day 5 post-exposure and thereafter until they became parasitemic or until day 30, volunteers were examined by an investigator. Two hundred high-power fields (1,000 $\times$) of thick blood films were examined for malaria. Parasite concentrations per μ l blood were quantitated by a modification of the method of Earle and Perez.^{7, 14} Daily, beginning on day 5, blood samples from each volunteer were established in culture. One ml of heparinized blood was centrifuged and the plasma and buffy coat removed. The cells were washed in serum-free RPMI 1640. Erythrocytes were resuspended in medium containing 10% heat-inactivated serum and incubated in a candle jar. The medium was changed on alternate days and Giemsa-stained thin films were examined weekly. Results from culture experiments were not considered in the clinical management of subjects.

Treatment of malaria

When parasites were detected on thick blood film, the subject was treated with 1,500 mg of oral chloroquine base given over 48 hr.¹⁵ The volunteer and blood films were examined twice daily until the volunteer was asymptomatic and 3 consecutive films were negative. Subjects who did not develop a blood-stage infection were given the same chloroquine regimen at day 30 post-exposure. Follow-up examinations were carried out 3 and 7 weeks after day 30.

RESULTS

Infection of volunteers

In the first experiment, 42% of the mosquitoes had sporozoites in their salivary glands, so that a total of 12 mosquitoes fed on the volunteers before all 5 had been exposed to an infected mosquito (1–4 mosquitoes/volunteer). Since only 3 of the 5 subjects developed malaria parasitemia after being bitten by 1 infected mosquito, a second experiment using 2 infected mosquitoes was conducted. In this experiment, 63% of the mosquitoes were infected. It required exposure to 16 mosquitoes for all 5 volunteers to have been bitten by 2 infected mosquitoes (2–6 mosquitoes/volunteer). Parasitemia developed in 2 of the 5 subjects who received bites from 2 infected mosquitoes (Table 1). Mosquitoes that fed on volunteers who did and did not become infected had salivary gland infections ranging from 2+ to 4+ (Table 1). Although the prepatent periods ranged from 14–16.5 days as detected by thick blood film, *P. falciparum* was cultured from blood taken on days 7–10 (Table 1); parasites were present in the circulation up to a week before they could be detected by thick blood film. The earliest blood sample from each subject that proved to be positive by culture required 21 days of culture before parasites were detected. Cultures of blood samples taken on later days had detectable parasites after as few as 4 days in culture.

Clinical findings

All volunteers who developed parasitemia also developed fever, chills, and myalgias; one subject also complained of headache, and another experienced a 6 hr episode of watery diarrhea (Table 2). None of the subjects developed hepatomegaly or splenomegaly. All symptoms and signs resolved within 48 hr of initiation of therapy, and only 1 of the subjects was sufficiently ill to require absence from duty. Subject no. 1 was the only subject hospitalized. He was hospitalized overnight and was treated only with acetaminophen in addition to chloroquine. His platelet count dropped from 225,000/ μ l to 126,000/ μ l 2 days after chloroquine treatment began. This subject also developed a mild leukopenia (3,300 WBC/ μ l) and had pyuria (5–10 WBC/HPF) and microscopic hematuria (5–10 RBC/HPF) for 8

TABLE 1

Malaria infections in normal humans subjected to the bites of infected mosquitoes: parasitological findings

Volunteer no.	No. of mosquitoes	Gland rate*	Prepatent period (days)†	Initial parasitemia (parasites μ l)	Maximum parasitemia (parasites μ l)	First day culture positive‡
1	1	3+	15	18	92	8
2	1	4+	14.5	11	11	7
3	1	2+	16.5	28	28	10
4	1	4+	NI§	NI	NI	NI
5	1	3+	NI	NI	NI	NI
6	2	4+ 4+	14	14	14	7
7	2	4+ 3+	14.5	11	11	8
8	2	4+ 3+	NI	NI	NI	NI
9	2	4+ 4+	NI	NI	NI	NI
10	2	4+ 4+	NI	NI	NI	NI

* 1+ = 1-10, 2+ = 11-100, 3+ = 101-1,000, 4+ = >1,000 sporozoites.

† The interval from inoculation to detection of parasitemia by thick blood film.

‡ The first day after exposure to infected mosquitoes that *P. falciparum* was present in blood established in culture.

§ NI = not infected.

hr without evidence of renal insufficiency. None of the other subjects developed abnormal complete blood counts or urinalyses. Liver function tests remained normal in all subjects.

DISCUSSION

In previous studies in which mosquitoes were infected by feeding on gametocyte cultures, volunteers were challenged with 5 infected mosquitoes.¹⁻⁷ At least 45 volunteers have been exposed to malaria using this method and, thus far, 100% (22/22) of unimmunized control subjects and 91% (21/23) of the immunized subjects have developed malaria parasitemia. In this study, exposure to 1 or 2 infected mosquitoes led to parasitemia in only 50% of the volunteers. These data therefore suggest that exposure to 5 infected mosquitoes may not represent an enormous spo-

rozoite challenge. This does not provide support for the hypothesis that the level of protective immunity induced by the first generation of malaria sporozoite vaccines could have been protective in a natural malarious environment but was not protective in the trials because an unrealistically large, overwhelming sporozoite challenge was used to test its protective efficacy.³⁻⁶

The NF54 strain of *P. falciparum* was used to challenge volunteers in previous studies. We used the 3D7 clone of the NF54 strain. The apparent lower rate of infectivity of the clone cannot be explained on the basis of gland rates. In the studies that reported gland rates,^{1-4,6} mean gland rates ranged between 1.95 and 3.8. The mean gland rate in this study was 3.6. It is possible that this lower infectivity is a reflection of the poor infectivity of the individual sporozoites of the clone compared to the parent strain. However, in re-

TABLE 2

Malaria infections in normal humans subjected to the bites of infected mosquitoes: clinical findings

Volunteer no.*	Incubation period†	Maximum temperature‡	Symptoms/signs§	Fever duration (days)	Duration of symptoms (days)
1	15	39.4	F, C, My, Ma, D	1.5	2
2	15	39	F, C, My	2	2
3	16	38.5	F, C, My, H	1.5	2
4	NI	NI	NI	NI	NI
5	NI	NI	NI	NI	NI
6	14	38.2	F, C, My, Ma	2	2
7	14	39	F, C, My	2	2
8	NI	NI	NI	NI	NI
9	NI	NI	NI	NI	NI
10	NI	NI	NI	NI	NI

* Subjects 1-5 received 1 bite from an infected mosquito, subjects 6-10 received 2 bites from infected mosquitoes.

† Days from exposure to infected mosquitoes to onset of clinical symptoms.

‡ Degrees C.

§ F = fever, C = chills, My = myalgias, Ma = malaise, D = diarrhea, H = headache, NI = not infected.

cent studies, 3 out of 3 subjects were successfully infected by 5 bites from mosquitoes carrying sporozoites of the 3D7 clone of NF54 (J. Egan, Walter Reed Army Institute of Research, Washington, DC, personal communication), while only 1 of 3 volunteers was infected when bitten by 2 mosquitoes infected with NF54 (L. Fries, Johns Hopkins University, Baltimore, MD, personal communication). This suggests that the infectivity of NF54 and the 3D7 clone of the NF54 strain are similar.

Nonetheless, it is difficult to explain the prolonged prepatent period of 14–16.5 days without invoking a biologic difference in the parasites. In previous studies,^{1–7} the prepatent period of controls has ranged from 7 to 11 days with a median of 10 days, and parasites have been first cultured from blood taken 6.5–7 days after exposure. In this study, parasites were first cultured from the blood 7–10 days after exposure, indicating that there has been no apparent change in the time required for the development of mature liver schizonts. The erythrocytic cycle of *P. falciparum* is ~48 hr, and each mature blood stage *P. falciparum* schizont has an average of 16 merozoites. If 10 of these merozoites are able to successfully infect other erythrocytes, one would expect a 10-fold increase in parasitemia every 2 days, a 100-fold increase in 4 days, and a 1,000-fold increase in 6 days. It is difficult to explain a 4–6 day delay in patency that would reflect a 100–1,000-fold decrease in the number of parasites based only on the difference between 2 and 5 infective bites. This is not the first description of a strain-dependent variation in prepatency. In a study comparing 3 *P. falciparum* strains (Panama, McLendon, and Santee-Cooper), mean prepatency periods were 10.3, 13, and 9.8 days, respectively.¹¹ In another study of 60 subjects, the prepatency periods of the strains used varied not only between strains but also over time.⁹ It remains unclear, however, whether the changes responsible for the differences in prepatent periods involve the injection of 100–1,000 times fewer sporozoites, a great reduction in the number of sporozoites reaching maturity in the liver, the production of far fewer infective merozoites, or the prolongation of the erythrocytic cycle.

Others have suggested that there may be a direct correlation between salivary gland load and number of sporozoites injected,^{5, 6} and that the short prepatent period in some studies could be explained on this basis. Among the volunteers

who became infected, there was a correlation between the numbers of sporozoites in the salivary glands and the prepatent periods and days until parasites were cultured from the blood. However, the salivary gland indices were equivalent in those mosquitoes that fed on the volunteers who did not become infected (Table 1). The important relationships among salivary gland index, number and infectivity of sporozoites injected, infection rate in the human population, and prepatency period remain unclear. It may be that salivary gland index will prove to be a poor predictor of the numbers of sporozoites injected.

More than 15 years ago, field studies indicated that most exposures to sporozoite-infected mosquitoes did not lead to transmission of malaria.¹⁶ More recent studies in Kenya have led to similar conclusions (C. Oster, Walter Reed Army Medical Center, Washington DC, personal communication). Our findings also support this view that while the entomologic inoculation rate reflects the transmission rate of malaria, it cannot be used to quantitate directly the transmission rate. Whether this is due to the failure of some mosquitoes to transmit sporozoites, or the lack of infectivity of the sporozoites which are transmitted, is unknown. A recent study indicates that, under laboratory conditions, the number of sporozoites ejected by infected mosquitoes can vary by 3 orders of magnitude.¹⁷

An important finding in this study, as in earlier studies, is that parasitemia was detected early so that appropriate chemotherapy could be rapidly initiated. Due to this rapid diagnosis, symptoms and signs were minimal and brief.

The criteria that predict the degree of infectivity of mosquitoes are not known. This leaves the vaccine developer uncertain about how many mosquito bites to use in a sporozoite vaccine trial. Too few bites by infected mosquitoes can leave one with uninfected control subjects and a loss of the ability to demonstrate protective efficacy. Too many bites by infected mosquitoes may deliver a large bolus of sporozoites, thereby overwhelming an immune response that would have provided protection against 1 bite received in the field. Five infective bites have been shown repeatedly to produce 100% infectivity in humans,^{1–7} and since 1 or 2 bites by infected mosquitoes do not produce consistent infections in volunteers, continuing to use 5 bites is reasonable.

Acknowledgments: The authors thank the volunteers who selflessly joined the study and who remained dependable, enthusiastic participants throughout. We also thank the nurses and corpsmen of wards 7 West and 6 East, NNMC for their support, Roy Trimmer for parasite cultures, Mary Leef for malaria antibody assays, Martha Sedegah for assistance during infection of the volunteers, Lynn Murphy for administrative support, Tom Coon for assistance in manuscript preparation, and Jose Salas for medical photography. The authors also thank John Beier and David Haynes for their very helpful reviews of the manuscript.

Financial support: Naval Medical Research and Development Command work unit number 63763A3M473750D808A0133.

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